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ORIGINAL ARTICLE

Effects of Smoking and Shared Epitope on the Production of Anti–Citrullinated Peptide Antibody in a Japanese Adult Population

CHIKASHI TERAO,[1] KOICHIRO OHMURA,[1] KATSUNORI IKARI,[2] TAKAHISA KAWAGUCHI,[1] MEIKO TAKAHASHI,[1] KAZUYA SETOH,[1] TAKEO NAKAYAMA,[1] SHINJI KOSUGI,[1] AKIHIRO SEKINE,[1] YASUHARU TABARA,[1] ATSUO TANIGUCHI,[2] SHIGEKI MOMOHARA,[2] HISASHI YAMANAKA,[2] RYO YAMADA,[1] FUMIHIKO MATSUDA,[1] AND TSUNEYO MIMORI,[1] ON BEHALF OF THE NAGAHAMA STUDY GROUP

Objective. **Anti–citrullinated peptide antibody (ACPA) and rheumatoid factor (RF) are markers to rheumatoid arthritis (RA). Smoking and shared epitope (SE) in HLA–DRB1 are associated with the production of these autoantibodies in RA. Detailed distribution and characterization of ACPA and RF in the general population have remained unclear. We aimed to evaluate positivity of ACPA and RF in a general Japanese population and to detect correlates, including genetic components.**

Methods. **ACPA and RF were quantified in 9,804 Japanese volunteers ages 30–75 years. Logistic regression analyses were performed to evaluate the effects of candidates of correlates on the autoantibody positivity. A genome-wide association study (GWAS) was performed using 394,239 single nucleotide polymorphisms for 3,170 participants, and HLA–DRB1 alleles were imputed based on the GWAS data.**

Results. **A total of 1.7% and 6.4% of subjects were positive for ACPA and RF, respectively, and the 2 markers showed a significant correlation ($P = 2.0 \times 10^{-23}$). Old age was associated with ACPA positivity ($P = 0.00062$). Sex, smoking, SE, and other candidates of correlates did not have significant effects. Interaction between smoking and SE positivity was not apparent, but smoking showed a significant association with high levels of ACPA ($P = 0.0019$).**

Conclusion. **ACPA and RF could be detected in 1.7% and 6.4% of the Japanese adult population without RA, respectively. ACPA and RF were suggested to share mechanisms even in healthy populations. Old age was associated with increasing ACPA positivity. While positivity of ACPA and RF was not associated with SE and smoking, an association between high ACPA and smoking was observed.**

INTRODUCTION

Rheumatoid factor (RF), an IgM autoantibody against the Fc fraction of IgG, is a serum marker of rheumatoid arthritis (RA) (1,2). In spite of its specificity to RA, RF appears in other diseases, especially connective tissue diseases, hepatic disorders, and even in healthy populations (3–9). Recently, anti–citrullinated protein antibody (ACPA) was found to show high specificity to RA and was able to distinguish RA from other connective tissue diseases with higher accuracy compared with RF (1,10). Although some studies reported functional pathogenicity of ACPA (11), pathogenicity and production mechanisms of ACPA and RF are largely unknown. Vigorous studies that address associations with the positivity and levels of ACPA and RF in patients with RA identified a wide range of factors. Some are disease-specific factors, such as disease

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[1]Chikashi Terao, MD, PhD, Koichiro Ohmura, MD, PhD, Takahisa Kawaguchi, MSc, Meiko Takahashi, PhD, Kazuya Setoh, MSc, Takeo Nakayama, MD, PhD, Shinji Kosugi, MD, PhD, Akihiro Sekine, PhD, Yasuharu Tabara, PhD, Ryo Yamada, MD, PhD, Fumihiko Matsuda, PhD, Tsuneyo Mimori, MD, PhD: Kyoto University, Kyoto, Japan; [2]Katsunori Ikari, MD, PhD, Atsuo Taniguchi, MD, PhD, Shigeki Momohara, MD, PhD, Hisashi Yamanaka, MD, PhD: Tokyo Women's Medical University, Tokyo, Japan.

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

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Significance & Innovations

- Positivity of anti–citrullinated peptide antibody (ACPA) in the general population is associated with aging and high C-reactive protein level.
- Smoking and shared epitope do not have comparable effect in the general population on the production of ACPA and rheumatoid factor (RF) as with patients with rheumatoid arthritis.
- Smoking may be associated with a high level of ACPA, even in healthy subjects.
- Correlates should be taken into account for RF and ACPA positivity in the general population. Novel findings of RF and ACPA production in general populations would provide clues to uncover the pathophysiology of the production of these autoantibodies.

activity and extraarticular symptoms (12–14) and others are disease–non-specific factors such as age, smoking, and common variants of HLA alleles (8,15–17). Smoking was shown to have an effect on the susceptibility to seropositive RA, especially in men (18). HLA–DRB1 is the strongest susceptibility locus to RA and is associated with ACPA or RF positivity in patients with RA (19). In particular, shared epitope (SE), an allelic group with a common amino acid pattern from the 70th to the 74th amino acid of the HLA–DRB1 protein (20), is strongly associated with RA susceptibility and production of ACPA and RF in patients with RA (15,17).

However, the distribution of these antibodies and whether the correlates are associated with positivity of ACPA or RF in the general population is largely unknown. There are no reports where ACPA levels were quantified and correlates of ACPA were analyzed in a large-scale study of healthy individuals. Although there are reports suggesting that the positivity of RF in healthy individuals is influenced by age and smoking in a European population (8,21–25), the positivity of RF and its correlates in healthy individuals is not known in Asian populations. If the likelihood of having RA based on positivity of ACPA or RF is different between subgroups with and without correlates, determining the distribution and correlates of ACPA and RF in a healthy population would lead to efficient screening to identify subjects at risk of RA. Moreover, determining the distribution and correlates would give clues for novel insights of mechanisms of production for ACPA and RF.

Here, we quantified circulating levels of ACPA and RF in 9,804 healthy Japanese subjects, identified prevalence, and estimated correlates, including genetic factors, of these 2 autoantibodies.

PATIENTS AND METHODS

Study population. This study was conducted as a part of the Nagahama Prospective Genome Cohort for Comprehensive Human Bioscience (The Nagahama Study) (26), a community-based prospective multiomics cohort study conducted by Kyoto University. A total of 9,804 volunteers in Nagahama City, Shiga Prefecture, Japan were recruited in this study from 2008 to 2010. All participants were asked to complete a detailed questionnaire about their present symptoms, present illness, past history of illness, family history, and smoking status. Written informed consent was obtained from all of the participants. This study was approved by Kyoto University Graduate School and Faculty of Medicine Ethics Committee.

Exclusion of samples. We excluded volunteers from the association studies if they had or have had autoimmune diseases. Individuals who were judged from their answers to the questionnaire to possibly have autoimmune diseases were also excluded from the analyses. As a result, a total of 9,575 subjects were recruited for the analysis.

RA patients. A total of 2,067 patients with RA in Tokyo Women's Medical University, whose age at onset, sex, and data of ACPA and RF were available, were registered in this study. A total of 1,237 patients with RA in Kyoto University were used for correlation analysis of genetic components.

Quantifying of circulating autoantibody. Serum samples were obtained from all the participants. ACPA was quantified as second-generation anti–cyclic citrullinated peptide (anti-CCP) antibody by MesaCup CCP enzyme-linked immunosorbent assay kit (Medical and Biological Laboratories) (27,28). IgM-RF was quantified by latex turbidimetric immunoassay, Iatro-RF II (Mitsubishi Kagaku Medience) (29). Both autoantibodies were quantified by SRL for healthy individuals and in Tokyo Women's Medical University for patients with RA. The cutoff levels of the autoantibodies were according to manufacturer's instructions (ACPA <4.5 units/ml, RF ≤ 20 IU/ml).

Candidates of correlates for ACPA and RF. Age, sex, smoking status, Brinkman index (BI; number of cigarettes a day $\times$ smoking years) as a quantitative measure of smoking, alcohol consumption, body mass index (BMI), and serum level of C-reactive protein (CRP) were selected as candidates of correlates for ACPA and RF. They were selected based on the previous reports of significant association between RA and smoking and a study from the US analyzing correlates of anti–nuclear antibody in the general population (30). We classified all the included participants into 5 groups according to their age at 10-year intervals. Logistic linear regression analysis or chi-square test was performed to analyze the influence of candidates of correlates on the positivity of autoantibodies. The effects of smoking in conditions with alcohol consumption were also analyzed.

Genome-wide association study (GWAS). GWAS was performed for 3,710 samples of participants who joined the Nagahama Study during 2008 to 2009. A series of BeadChip DNA array was used for the genotyping and

several samples were repeatedly genotyped using different arrays. All samples were scanned by at least 1 of 3 arrays, namely, Illumina HumanHap610Quad, Omni2.5-4, and Omni2.5-8 (see Supplementary Table 1, available in the online version of this article at http://onlinelibrary.wiley.com/doi/10.1002/acr.22385/abstract). A total of 394,239 markers that were common across the 3 arrays were used for the current study. Genotyping quality was controlled by excluding single-nucleotide polymorphisms (SNPs) with a call rate below 95%, with minor allele frequency below 5%, and deviating from Hardy-Weinberg equilibrium ($P < 1.0 \times 10^{-7}$). Excluded from the analysis were 162 samples with a call rate <95%, 295 individuals estimated to have kinship within this population (PI-hat more than 0.35), and 7 ancestry outliers identified by principal component analysis, with HapMap Phase 2, release 28, data set as reference. A total of 83 individuals were excluded, because of having or being suspected of having connective tissue diseases from their answers to the questionnaire. As a result, 3,170 samples were analyzed for GWAS. Logistic regression analyses were performed by using positivity of ACPA and RF as dependent variables, each SNP as an independent variable, and age and sex as covariates.

HLA imputation. Alleles for HLA–DRB1 were imputed based on genotypes in the GWAS by using HLA–IMP2 (31). We imputed HLA–DRB1 alleles for 589 patients with RA for a test set as reported previously (28). Imputed HLA–DRB1 alleles were compared with genotyped HLA–DRB1 alleles and an algorithm for determining HLA–DRB1 alleles was established (See Supplementary Appendix A, available in the online version of this article at http://onlinelibrary.wiley.com/doi/10.1002/acr.22385/abstract). Next, HLA–DRB1 alleles were determined based on the same algorithm for 932 healthy individuals as described previously (32), and compared with the genotyped HLA–DRB1 alleles. The algorithms for HLA–DRB1 based on imputation provided more than 93.5% of sensitivity and 99.8% of specificity for SE. HLA–DRB1 alleles were inferred for the 3,170 individuals in the current GWAS using the same algorithm.

Correlation analysis. Effect sizes of SNPs in the logistic regression analysis for the autoantibody positivity in the healthy population were compared with those in the association study for RA susceptibility, recruiting 1,237 cases and 2,087 controls in Kyoto University and previously described elsewhere (19,32). The 259,249 SNPs that were common across the current study and the previous study were pruned by linkage disequilibrium of $r^2 > 0.3$. As a result, there were 82,445 SNPs remaining for further analysis. Correlation analysis was performed by using Pearson's correlation coefficients with 8 intervals, according to the P values in each study.

Power analysis. Power analysis was performed by an online power calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/).

Statistical analysis. Logistic regression analyses in genetic studies were performed by Plink, version 1.07 (33). Other statistical analyses were performed using the R statistic system (http://www.R-project.org) or SPSS (version 18). We regarded P values less than 0.005 as significant to assess correlations in a conservative manner. A stringent cutoff value of $P < 5 \times 10^{-8}$ was adopted for the GWAS.

RESULTS

Characteristics of ACPA and RF. In the current study, 1.7% and 6.4% of the study population (n = 9,575) showed positive ACPA and RF, respectively (Tables 1 and 2). The distribution of titers is shown in Table 1. We also found 0.44% of subjects being positive for both ACPA and RF, and a significant association between ACPA and RF positivity ($P = 2.0 \times 10^{-23}$ in chi-square test [odds ratio (OR) 5.19 (95% confidence interval [95% CI] 3.62–7.44)]) (see Supplementary Table 2, available in the online version of this article at http://onlinelibrary.wiley.com/doi/10.1002/acr.22385/abstract). The individuals who were positive for both ACPA and RF showed a significant correlation of the titers of these autoantibodies ($\rho = 0.60$) (see Supplementary Figure 1, available in the online version of this article at http://onlinelibrary.wiley.com/doi/10.1002/acr.22385/abstract). When we analyzed effects of candidates of correlates on positivity of these autoantibodies, we did not observe a significant difference in positivity for RF and ACPA between men and women. We found that ACPA positivity increased with respect to older age ($P = 0.00045$ in logistic linear regression analysis), especially for those in their 70s ($P = 0.00062$) (Table 2). While people in their 50s showed an increase of RF positivity ($P = 5.4 \times 10^{-5}$) (Table 2), no linear effect of age on RF positivity was observed ($P = 0.093$ in logistic linear regression analysis). The associations between age and ACPA or increase of RF for those in their 50s were observed mainly in women (see Supplementary Table 3, available in the online version of this article at http://onlinelibrary.wiley.com/doi/10.1002/acr.22385/abstract). Next, BMI, smoking, alcohol consumption, and serum level of CRP were analyzed for associations with ACPA and RF positivity. While we did not find

Table 1. Distribution of titers in ACPA and RF in the general population*

	No.	Ratio, %
ACPA (units/ml)		
<4.5	9,408	98.3
4.5–13.5	100	1.0
>13.5–45	33	0.3
>45	34	0.4
RF (IU/ml)		
≤20	8,961	93.6
20–60	486	5.0
>60–200	87	0.9
>200	41	0.4

* ACPA = anti–citrullinated peptide antibody; RF = rheumatoid factor.

Table 2. Distribution and correlates of ACPA and RF in the general population*

		ACPA			RF		
	No.	Positivity, %	*P*†	OR (95% CI)†	Positivity, %	*P*†	OR (95% CI)†
All subjects	9,575	1.7	–	–	6.4	–	–
Sex							
Male	3,168	1.7	–	Reference	5.7	–	Reference
Female	6,407	1.8	0.84	1.04 (0.74–1.45)	6.8	0.040	1.21 (1.00–1.45)
Age, years							
30–39	2,315	1.3	–	Reference	5.4	–	Reference
40–49	1,339	1.3	0.94	0.98 (0.53–1.80)	5.8	0.67	1.06 (0.79–1.44)
50–59	1,886	1.8	0.23	1.35 (0.82–2.25)	8.7	5.4×10^{-5}	1.64 (1.29–2.10)‡
60–69	3,012	1.8	0.12	1.43 (0.90–2.26)	6.3	0.13	1.20 (0.95–1.52)
70–75	1,023	3.0	0.00062	2.46 (1.45–4.15)‡	5.7	0.67	1.07 (0.77–1.49)
BMI, kg/m^2							
18.5–25	6,876	1.8	–	Reference	6.6	–	Reference
<18.5	902	1.2	0.37	0.75 (0.39–1.42)	6.7	0.88	1.02 (0.76–1.37)
25–30	1,567	2.0	0.71	1.08 (0.72–1.63)	5.5	0.15	0.84 (0.66–1.07)
≥30	230	1.3	0.66	0.77 (0.24–2.51)	7.0	0.72	1.10 (0.65–1.86)
Smoking							
Never	6,219	1.7	–	Reference	6.7	–	Reference
Ex-smoker	1,961	2.0	0.21	1.36 (0.83–2.20)	5.8	0.97	1.00 (0.77–1.31)
Active	1,395	1.6	0.49	1.22 (0.69–2.16)	6.2	0.46	1.11 (0.83–1.49)
0 < BI ≤ 200	1,056	1.5	0.65	1.14 (0.65–2.02)	5.0	0.23	0.83 (0.61–1.13)
200 < BI ≤ 600	1,254	1.7	0.32	1.34 (0.75–2.39)‡	5.3	0.82	1.04 (0.75–1.43)
600 < BI	1,044	2.2	0.32	1.42 (0.71–2.83)‡	7.5	0.018	1.58 (1.08–2.30)‡
Alcohol§							
Never or past	3,193	2.1	–	Reference	6.3	–	Reference
Current, light	1,883	1.8	0.66	0.91 (0.58–1.41)	7.3	0.049	1.26 (1.00–1.60)
Current, moderate/heavy	3,396	1.3	0.025	0.60 (0.38–0.95)§	5.8	0.84	1.02 (0.81–1.29)
CRP, mg/dl							
<0.1	1,587	1.1	–	Reference	5.4	–	Reference
0.1–0.3	3,350	1.7	0.27	1.36 (0.78–2.37)	6.4	0.17	1.20 (0.92–1.58)
>0.3–1.0	3,235	1.5	0.64	1.14 (0.64–2.06)	6.4	0.30	1.16 (0.87–1.53)
≥1.0	1,403	3.0	0.0078	2.26 (1.22–4.17)‡	7.5	0.0087	1.53 (1.11–2.12)‡

* ACPA = anti–citrullinated peptide antibody; RF = rheumatoid factor; OR = odds ratio; 95% CI = 95% confidence interval; BMI = body mass index; BI = Brinkman's Index; CRP = C-reactive protein.
† Logistic regression analysis adjusting for sex and age (statistics for BMI, alcohol, smoking, and CRP level).
‡ Suggestive or significant associations mentioned in the main text.
§ Those that drink more than once a week are categorized as moderate/heavy.

any significant associations for BMI, smoking, and alcohol consumption (Table 2), high alcohol consumption showed a suggestive protective effect with ACPA positivity, consistent with the previous report from European populations showing a protective effect of alcohol consumption against ACPA-positive RA (34). Smoking showed a suggestive dose-dependent effect on ACPA production, and this effect was strengthened in condition with alcohol consumption (Table 2 and Supplementary Table 4, available in the online version of this article at http://onlinelibrary.wiley.com/doi/10.1002/acr.22385/abstract). A high level of CRP showed suggestive associations with ACPA and RF positivity (P = 0.0078 and 0.0087, respectively) (Table 2). Because smoking is the established environmental risk factor for seropositive RA, especially in men, we separately analyzed effects of smoking on ACPA and RF production in men and women. As a result, while we found a slight increase of positivity in male ever-smokers, the associations did not reach a significant level and the ORs were much lower than those for seropositive RA (Table 3).

The linear increase of ACPA positivity according to ages of individuals raised the possibility that the positive likelihood ratio (PLR) of having RA based on ACPA positivity differed according to the age groups. To address this point, we collected ACPA data from 2,067 patients with RA whose data on age at onset were available and calculated the PLR of having RA based on ACPA positivity. As a result, we found that the PLR of having RA decreased according to age (Supplementary Figure 2A, available in the online version of this article at http://onlinelibrary.wiley.com/doi/10.1002/acr.22385/abstract). In particular, the group age >70 years demonstrated a significantly lower PLR of RA than the group ages 30–39 years (P = 0.0033) (Supplementary Figure 2A, available in the online version of this article at http://onlinelibrary.wiley.com/doi/10.1002/ acr.22385/abstract). When we analyzed RF positivity in the same manner, the PLR decreased in the group ages 50–59 years in comparison to the group ages 30–39 years, reflecting an increase of RF positivity in the general population (P = 0.0013) (Supplementary Figure 2B, available in the online version of this article

Table 3. Lack of significant association between smoking and ACPA or RF in men and women*

All subjects	ACPA				RF			
	No.	Positivity, %	*P*†	OR (95% CI)†	No.	Positivity, %	*P*†	OR (95% CI)†
Men								
Never	791	1.3	–	Reference	791	4.7	–	
Ex-smoker	1,399	2.1	0.25	1.62 (0.71–3.66)	1,399	5.8	0.55	Reference
Active	978	1.5	0.38	1.50 (0.60–3.74)	978	6.3	0.13	1.14 (0.74–1.76)
Ever (Ex and Active)	2,377	1.9	0.27	1.54 (0.71–3.36)	2,377	6.0	0.28	1.42 (0.91–2.23)
0 < BI ≤ 200	436	1.6	0.67	1.31 (0.38–4.49)	436	5.0	0.72	1.24 (0.84–1.84)
200 < BI ≤ 600	943	1.5	0.53	1.35 (0.53–3.45)	943	4.8	0.99	1.11 (0.62–2.00)
600 < BI	981	2.3	0.27	1.60 (0.70–3.69)	981	7.6	0.10	1.00 (0.62–1.62)
Women								1.46 (0.93–2.29)
Never	5,428	1.8	–	Reference	5,428	6.9	–	
Ex-smoker	562	1.8	0.60	1.22 (0.59–2.50)	562	5.7	0.30	Reference
Active	417	1.7	0.51	1.31 (0.59–2.91)	417	6.0	0.53	0.80 (0.52–1.22)
Ever (Ex and Active)	979	1.7	0.44	1.25 (0.71–2.21)	979	5.8	0.27	0.86 (0.53–1.38)
0 < BI ≤ 200	620	1.5	0.73	1.13 (0.55–2.35)	620	5.0	0.063	0.83 (0.59–1.15)
200 < BI ≤ 600	311	2.3	0.20	1.68 (0.76–3.72)	311	7.1	0.55	0.65 (0.42–1.02)
600 < BI	41	0	0.98	NA	41	7.3	0.73	1.15 (0.72–1.84)

* ACPA = anti–citrullinated peptide antibody; RF = rheumatoid factor; OR = odds ratio; 95% CI = 95% confidence interval; BI = Brinkman's Index; NA = not applicable.
† *P* values and ORs in logistic regression analysis using age and alcohol drinking as covariates.

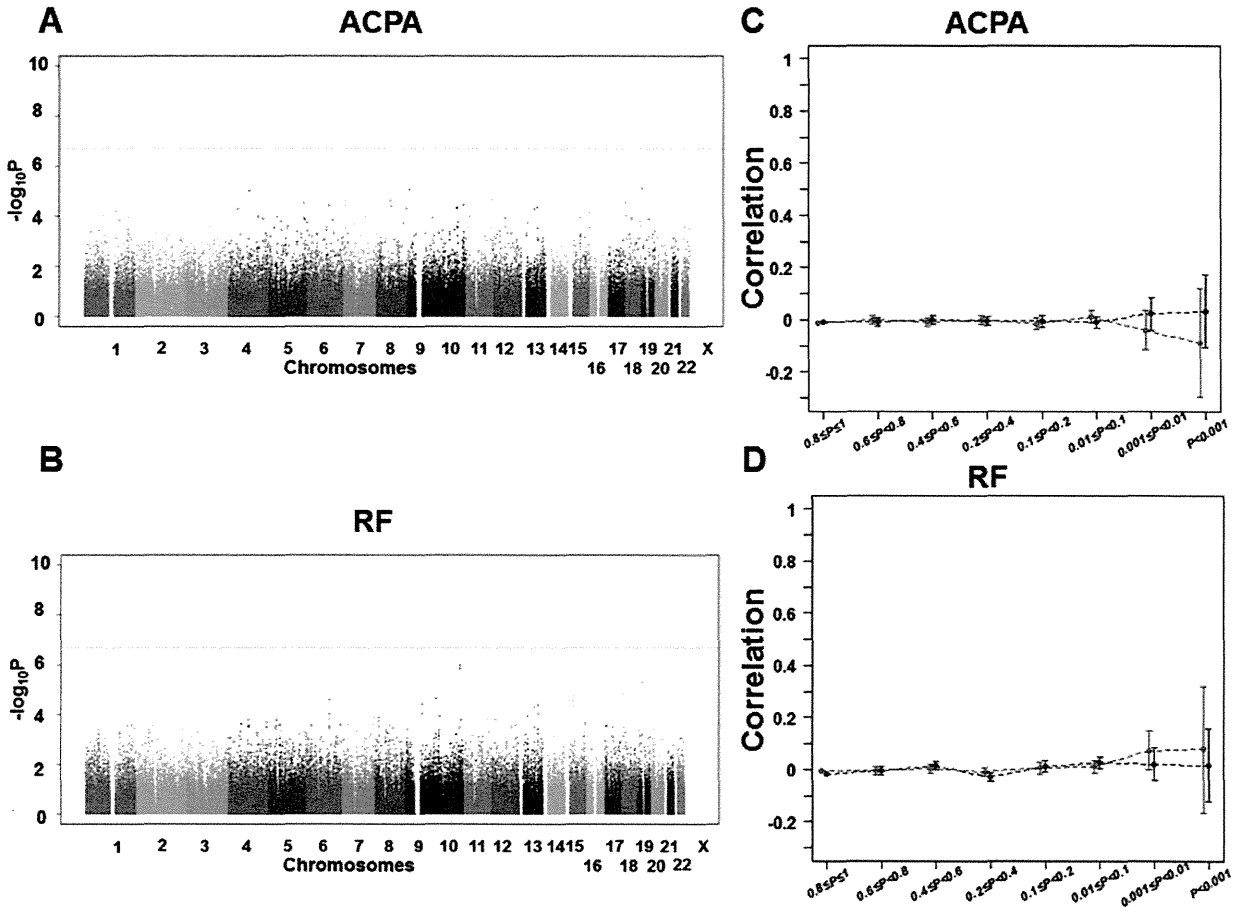

Figure 1. Genetic associations of single-nucleotide polymorphism (SNPs) and anti–citrullinated peptide antibody (ACPA) or rheumatoid factor (RF) positivity. Manhattan plot is shown for positivity of ACPA (**A**) or RF (**B**). No SNPs showed significant associations with positivity of ACPA or RF. Limited correlations of odds ratios in the SNPs of genome-wide association studies between RA susceptibility and positivity of ACPA (**C**) or RF (**D**). Blue broken lines indicate SNPs with *P* values in the range of x-axis for positivity of ACPA (**C**) or RF (**D**). Red broken lines indicate SNPs with *P* values in the range of x-axis for RA susceptibility. SNPs are pruned by $r^2 < 0.3$. The error bars indicate 95% confidence interval.

Table 4. Lack of significant associations between positivity of ACPA or RF and combination of SE and smoking in the general population*

	No.	ACPA			RF		
		Positivity, %	*P*†	OR (95% CI)†	Positivity, %	*P*†	OR (95% CI)†
SE (-)	1,935	1.7	–	Reference	6.0	–	Reference
SE (+)	1,235	1.9	0.82	0.93 (0.47–1.81)	6.8	0.95	1.01 (0.71–1.44)
All subjects							
SE (-) nonsmoker	1,230	1.8	–	Reference	6.4	–	Reference
SE (-) ex-smoker	373	2.4	0.063	2.83 (0.94–8.46)	4.3	0.16	0.58 (0.27–1.24)
SE (-) current smoker	332	0.6	0.40	0.50 (0.10–2.47)	6.0	0.64	0.84 (0.41–1.74)
SE (-) BI	–	–	0.045	1.13 (1.00–1.27)	–	0.24	1.05 (0.97–1.13)
SE (+) nonsmoker	772	2.1	0.50	1.32 (0.59–2.98)	7.0	0.89	0.97 (0.63–1.48)
SE (+) ex-smoker	265	1.9	0.65	1.41 (0.31–6.33)	7.2	0.75	0.88 (0.41–1.89)
SE (+) current smoker	198	1.0	0.68	0.71 (0.14–3.63)	5.6	0.44	0.68 (0.25–1.82)
SE (+) BI	–	–	0.26	0.84 (0.62–1.14)	–	0.48	0.96 (0.86–1.07)
Male							
SE (-) smoking (-)	184	1.1	–	Reference	6.0	–	Reference
SE (-) smoking (+)	461	1.5	0.65	1.45 (0.30–7.07)	5.2	0.51	0.75 (0.33–1.75)
SE (-) BI	–	–	0.38	1.06 (0.93–1.20)	–	0.24	1.05 (0.97–1.14)
SE (+) smoking (-)	93	3.2	0.25	2.97 (0.48–18.44)	2.2	0.20	0.36 (0.08–1.73)
SE (+) smoking (+)	334	1.8	0.53	1.69 (0.33–8.52)	6.0	0.51	0.74 (0.31–1.81)
SE (+) BI	–	–	0.33	0.92 (0.78–1.09)	–	0.23	0.93 (0.83–1.05)
Female							
SE (-) smoking (-)	1,046	1.9	–	Reference	6.6	–	Reference
SE (-) smoking (+)	244	1.6	1.00	1.00 (0.32–3.15)	4.9	0.28	0.61 (0.25–1.50)
SE (-) BI	–	–	–	–	–	0.93	1.01 (0.80–1.27)
SE (+) smoking (-)	679	1.9	0.81	1.10 (0.53–2.26)	7.7	0.84	1.05 (0.65–1.69)
SE (+) smoking (+)	129	0.8	0.44	0.44 (0.06–3.43)	7.8	0.81	0.88 (0.30–2.61)
SE (+) BI	–	–	–	–	–	0.24	1.18 (0.90–1.56)

* ACPA = anti–citrullinated peptide antibody; RF = rheumatoid factor; SE = shared epitope; OR = odds ratio; 95% CI = 95% confidence interval; BI = Brinkman's Index.
† Logistic regression analysis adjusting for age, sex, and alcohol consumption or age and alcohol consumption for the analysis of all subjects and men or women, respectively. Results of logistic regression analysis adjusting for only age were shown for ACPA analyses of men, women, and subgroup with <5 subjects positive for ACPA. Linear regression analysis of BI was applied for subsets with >5 smoking subjects.

at http://onlinelibrary.wiley.com/doi/10.1002/acr.22385/abstract).

Genetic components. Next we performed a GWAS in 3,170 healthy subjects to estimate common variants associated with positivity of ACPA or RF. The GWAS did not show population stratification in both studies ($\lambda \leq 1.00$). Both GWAS did not demonstrate significant associations ($P < 5 \times 10^{-8}$) in any markers, including the HLA locus (Figures 1A and B). Due to limitations of sample sizes with positive ACPA or RF in the current study and the possibility of multiple variants with low effect sizes associated with the phenotypes, it could be that truly associated SNPs were enriched in the SNP group with suggestive associations, even if we did not find markers with significant associations. As previous genetic studies have suggested that SNPs with low to middle effect sizes contribute to RA phenotypes beyond ethnicity (19) even if they did not show significant associations, we hypothesized that multiple common variants with low to middle effect sizes contributing to RA would be associated with ACPA or RF production in the general population and vice versa. Therefore, we performed correlation analyses to compare the effect sizes of SNPs between the RA study and the 2 GWASs. We used data of the RA GWAS that recruited 1,237 cases and 2,087 controls in Kyoto University, which was a part of a previously published meta-analysis in a Japanese population (19). As a result, we did not find significant correlations between RA susceptibility and RF or ACPA positivity, even in a set of SNPs showing *P* values less than 0.001 in each GWAS ($P \geq 0.40$) (Figure 1C and D).

HLA–DRB1 and positivity of ACPA and RF. Since the association between SE and positivity of ACPA and RF in patients with RA is well established, we analyzed whether these associations were observed in the general population. We imputed HLA–DRB1 alleles in the 3,170 individuals by HLA–IMP2 based on the genome-scanning data (details shown in Patients and Methods). Imputation of SE showed more than 93.5% of sensitivity and 99.8% of specificity for the genotyped SE in the 2 independent sets. The association studies showed that SE was not significantly associated with ACPA and RF positivity ($P = 0.82$ and 0.95, respectively) (Table 4). Because previous studies showed that associations between SE and positivity for ACPA and RF in patients with RA were strengthened in the smoking population, we classified our subjects into 3 groups according to smoking status and assessed effects of

Table 5. Significant associations between high ACPA level and smoking status or smoking quantity*

		RF high			ACPA high		
	No.	Positivity, %	*P*†	OR (95% CI)†	Positivity, %	*P*†	OR (95% CI)†
Smoking (-)	6,219	1.2	–	Reference	0.6	–	Reference
Smoking (+)	3,356	1.5	0.57	1.16 (0.70–1.90)	1.0	0.0019	3.01 (1.50–6.03)‡
Smoking BI	–	–	0.0066	1.08 (1.02–1.15)‡	–	0.00011	1.14 (1.07–1.22)‡
SE (-)	1,935	1.3	–	Reference	0.5	–	Reference
SE (+)	1,235	1.0	0.35	0.75 (0.42–1.37)	0.6	0.46	1.33 (0.62–2.84)

* Nonsmoking subjects without rheumatoid factor (RF) or anti–citrullinated peptide antibody (ACPA) were set as reference for the analysis of smoking. Subjects without shared epitope (SE) and RF or ACPA were set as reference for the analysis of SE. OR = odds ratio; 95% CI = 95% confidence interval; BI = Brinkman's Index.
† Logistic regression analysis adjusting for age, sex, and alcohol drinking, or age and sex for the analysis of smoking or SE, respectively.
‡ Suggestive or significant associations mentioned in the main text.

SE. We did not find significant associations in any of the 3 groups (nonsmoking, ex-smoking, and currently smoking) (Table 4) and smoking quantity. As a previous study suggested that male subjects with SE are more sensitive to smoking in ACPA production (35), men and women were analyzed separately. We found an increase of ACPA positivity in SE-positive groups both for male nonsmoking and smoking groups, but the associations did not reach the significant level (Table 4).

Association between high level of ACPA and smoking or SE. Because the distribution of ACPA or RF levels in subjects positive for these antibodies is different between healthy people and patients with RA (see Supplementary Table 5, available in the online version of this article at http://onlinelibrary.wiley.com/doi/10.1002/acr.22385/abstract), we focused on those individuals with high levels of ACPA or RF. While the decreased number of positive subjects made it difficult to conclude the association, we observed a significant association between smoking and high levels of ACPA with a comparable effect size to patients with RA ($P = 0.0019$, OR 3.01 [95% CI 1.50–6.03]) (Table 5). Further, smoking showed a dose-dependent association with high levels of ACPA ($P = 0.00011$). Although smoking did not show association with high levels of RF, we observed a suggestive dose-dependent effect of smoking on high RF production ($P = 0.0066$) (Table 5). We found that the association trend between smoking and high ACPA levels was enhanced in male subjects (Supplementary Table 6, available in the online version of this article at http://onlinelibrary.wiley.com/doi/10.1002/acr.22385/abstract). On the contrary, we did not find associations between SE and high levels of RF or ACPA.

DISCUSSION

In the current study, we showed positivity of ACPA and RF in the general population, analyzed correlates of these autoantibodies, and assessed genetic effects alone and in combination with smoking. This is the first study to quantify ACPA and RF in a large-scale healthy population to assess correlates. Although the positivity of ACPA in this study was comparable to that in the previous Turkish study (1.0% in 941 subjects) (36), the positivity of RF was slightly higher than those in the previous study that were highly variable (21,25,36). This high positivity of RF in the current study may be explained by the high proportion of female subjects who showed a suggestive increase of RF positivity compared to men, and the high proportion of subjects in their 50s who showed the highest positivity of RF among the groups. The 201 subjects excluded due to possibly having connective tissue diseases showed positivity of 27.9% and 34.8% for ACPA and RF, respectively, reflecting that many of them have rheumatic diseases (data not shown). Considering the prevalence of RA patients in the Japanese population (0.5–1.0%), the frequency of excluded subjects in the current study (approximately 2% of study subjects) seems reasonable. Therefore, it is less likely that patients with RA were missed for exclusion and enriched in the remaining subjects. The cutoff values of 26 and 45 IU/ml for RF would give 95% and 98% specificity in the current study, respectively. ACPA showed more than 98% specificity with the current cutoff value.

The positivity of ACPA and RF showed correlations even in the general population. Although the OR of being positive for both autoantibodies is lower than that in patients with RA (the 2,067 patients in this study: OR 24.79 [95% CI 17.84–34.45]; data not shown), the titers in subjects positive for both autoantibodies also showed a good correlation. These might suggest that both autoantibodies share common genetic and/or environmental risk factors.

ACPA and RF positivity did not show strong association with sex. As approximately 80% of RA patients are women (37), the lack of association suggests that factors other than sex are essential to produce ACPA and RF. We detected an age-dependent increase of ACPA positivity. This result corresponds to a previous report suggesting that detectable levels of antibodies against fillagrin, one of the important targets of citrullination of RA, tended to be found in the older population (38). RF showed an inverse U pattern in association with age. Menopausal term seems to correspond to the peak of RF positivity in female subjects. However, when we divided female subjects ages 50–59 years into 2 groups based on menopause, we did not find a significant difference in the positivity of RF ($P = 0.31$ and OR 0.78; data not shown). The same tendency of

increase of RF positivity in men cannot be explained by menopause. A prospective study to follow the same participants to observe the level of RF and compare RF positivity before and after menopause may lead to more clues for mechanisms underlying RF production. Previous studies showed that the elderly population has high frequency of RF in Europe and the US (24,25). Men showed the suggestive association between aging and RF production in this study. The difference between populations may suggest that different environmental factors play a role in autoantibody production. In fact, a recent twin study analyzing ACPA revealed that large parts of variance of ACPA can be explained by nonshared environmental factors (39). Different PLRs suggest that when individuals were incidentally found to be positive for these autoantibodies, ACPA in particular, the likelihood of having RA or having risk of RA is different based on age.

Analysis of candidates of correlates for ACPA and RF resulted in a positive association between high levels of CRP and ACPA or RF positivity. While this suggests an association between the production of these autoantibodies with preclinical inflammation, the current cross-sectional study could not conclude whether the production is a cause or a result. Other candidates for correlates were not associated with ACPA or RF. Many studies recruiting RA patients have shown that smoking is a strong environmental factor to produce ACPA and to develop RA (17,35). In our study, the associations between smoking and ACPA or RF positivity are not significant, while we observed significant or suggestive dose-dependent effects of smoking on high levels of ACPA or RF, respectively. A previous European study showed an association between active smoker and RF production in a healthy population (23). Since the median BI was 370 in the ever-smokers in the current study, equivalent to 1 pack a day for approximately 18 years, the less amount of smoking may contribute to a low effect of smoking status on autoantibody production in the current study. Previous studies revealed that ever smoking showed an OR of approximately 3 for male seropositive RA and 1.3 for female (18). Based on the seropositivity of nonsmokers, the current study is powered 100% in men and 68% in women to detect the effects with an OR of 3 and 1.3, respectively, at a level of $P = 0.05$. These results suggest that smoking is not associated with production of RF and ACPA at low levels but may be associated with the production of these antibodies at high levels in a healthy population. Although the current study cannot conclude that the association between smoking and high ACPA or RF is true due to the limited number of positive subjects, it is feasible to increase the number of healthy subjects. It will also be interesting to analyze smoking effects on low ACPA or RF levels in patients with RA. Isotypes of RF and ACPA were not quantified in the current study. Detailed classification of RF and ACPA would reveal specific associations of correlates, especially smoking.

While a recent twin study showed that heritability of ACPA was 0.23 (39), GWAS for positivity of RF or ACPA resulted in no significant signals, including the HLA locus. Our study had a power of 0.99 to detect an SNP with allele frequency of 0.4 (SE-positive ratio in healthy subjects) and an OR of 2.0 associated with 7% of frequent phenotype at a level of $P = 0.00001$. Our study also had a power of 0.42 to detect an SNP associated with 1.6% frequent phenotype at a level of $P = 0.01$. The imputed SE showed an OR of 1.15 for both ACPA and RF. Considering an OR of 2.0–3.0 in patients with RA for positivity of RF or ACPA in the previous studies, the current study indicates that SE was not similarly associated with ACPA and RF production in the general population as in RA patients. Furthermore, we did not observe associations between SE and high ACPA or RF. ACPA and RF production may need other factors than SE, such as chronic inflammatory stimulation. While the male population showed suggestive associations between SE and ACPA production, the limited number of the positive subjects did not allow us to draw any conclusions. Common direction of SE and smoking for ACPA production in men suggests that men are more sensitive to these risk factors than women. Although previous studies reported that HLA–DRB1*09:01 had a lowering effect of ACPA in the Japanese (27), we did not find a significant effect of *09:01 on ACPA positivity (Supplementary Table 7, available in the online version of this article at http://onlinelibrary.wiley.com/doi/10.1002/acr.22385/abstract). HLA–DR13, especially DRB1*13:01, shows a negative association with ACPA-positive RA in the European population (40). Although we did not find ACPA-positive subjects with DRB1*13:01 in the current study (Supplementary Table 7, available in the online version of this article at http://onlinelibrary.wiley.com/doi/10.1002/acr.22385/abstract), low frequency of DRB1*13:01 made it difficult to conclude the association between ACPA production and DRB1*13:01. No associations were detected between DR13 and ACPA production either. The negative results of genetic correlation analyses suggest that RA susceptibility and ACPA or RF production in the general population share limited genetic components in spite of wide confidence intervals of SNPs due to low power of the current study.

Because disease-specific autoantibodies, including ACPA and RF, were shown to appear several years before the diagnosis of the diseases (21,41–45), it will be interesting to follow the current study population to observe whether or not they will develop RA. It will also be very interesting to validate our results in other populations and compare the associations among the different populations.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be submitted for publication. Dr. Terao had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Terao, Ohmura, Ikari, Kawaguchi, Takahashi, Setoh, Nakayama, Kosugi, Sekine, Tabara, Taniguchi, Momohara, Yamanaka, Yamada, Matsuda, Mimori.

Acquisition of data. Terao, Ohmura, Ikari, Kawaguchi, Takahashi, Setoh, Nakayama, Kosugi, Sekine, Tabara, Taniguchi, Momohara, Yamanaka, Yamada, Matsuda, Mimori.
Analysis and interpretation of data. Terao, Kawaguchi.

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Association Between Antinuclear Antibodies and the HLA Class II Locus and Heterogeneous Characteristics of Staining Patterns

The Nagahama Study

Chikashi Terao, Koichiro Ohmura, Ryo Yamada, Takahisa Kawaguchi, Masakazu Shimizu, Yasuharu Tabara, Meiko Takahashi, Kazuya Setoh, Takeo Nakayama, Shinji Kosugi, Akihiro Sekine, Fumihiko Matsuda, and Tsuneyo Mimori
on behalf of the Nagahama Study Group

Objective. **While antinuclear antibodies (ANAs) are observed in healthy populations as well as in patients with autoimmune diseases such as systemic lupus erythematosus (SLE), the detailed genetic background of ANAs has remained unclear. We undertook this study to identify the genetic determinants of ANAs in the general population in order to elucidate the underlying mechanisms of ANA production and to distinguish disease susceptibility genes from ANA production genes.**

Methods. **A total of 9,575 Japanese volunteers were registered, and their ANA levels were quantified using indirect immunofluorescence to analyze correlates of ANA positivity. Genetic studies were performed using 7,148 of the 9,575 subjects. We performed a genome-wide association study using 3,185 subjects genotyped for 303,506 single-nucleotide polymorphisms (SNPs), followed by a replication study of 3,963 subjects. HLA–DRB1 and HLA–DQB1 alleles were imputed, and associations between ANA positivity and the SNPs or the HLA alleles associated with SLE were analyzed.**

Results. **Female sex and old age were associated with ANA positivity, except for the nucleolar pattern. The T allele of rs2395185 in the HLA locus, which was in moderate linkage disequilibrium with HLA–DRB1*0405, was significantly associated with ANA positivity ($P = 1.3 \times 10^{-11}$). The T allele of rs2395185 displayed increasing effects on the frequency of speckled and homogeneous patterns ($P = 7.5 \times 10^{-12}$ and $P = 2.2 \times 10^{-11}$, respectively) but decreasing effects on the frequency of the nucleolar pattern ($P = 0.0045$). The 7 SNPs and 4 HLA–DRB1 alleles associated with SLE did not display strong associations with ANA positivity.**

Conclusion. **SNP rs2395185 linked with HLA–DRB1*0405 is a genetic determinant of ANA production in the Japanese population. Overlapping of loci for susceptibility to SLE and to ANA positivity was limited. The nucleolar pattern showed different associations from other staining patterns, both with correlates of ANA positivity and with the HLA locus.**

Antinuclear antibodies (ANAs) are autoantibodies that recognize various nuclear and cytoplasmic proteins, and they are frequently observed in patients with a broad range of diseases including systemic lupus erythematosus (SLE), hepatic disease, malignant disease, lung disease, and a variety of infections (1–6). The distribution patterns of fluorescent types of ANAs (such as speckled, homogeneous, nucleolar, or discrete speck-

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Chikashi Terao, MD, PhD, Koichiro Ohmura, MD, PhD, Ryo Yamada, MD, PhD, Takahisa Kawaguchi, MSc, Masakazu Shimizu, PhD, Yasuharu Tabara, PhD, Meiko Takahashi, PhD, Kazuya Setoh, MSc, Takeo Nakayama, MD, PhD, Shinji Kosugi, MD, PhD, Akihiro Sekine, PhD, Fumihiko Matsuda, PhD, Tsuneyo Mimori, MD, PhD: Kyoto University, Kyoto, Japan.

Address correspondence to Chikashi Terao, MD, PhD, Center for Genomic Medicine, Kyoto University Graduate School of Medicine, Shogoin-Kawahara-cho 54, Sakyo-ku, Kyoto 606-8507, Japan. E-mail: a0001101@kuhp.kyoto-u.ac.jp.

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led patterns) also provide useful information for differential diagnosis (7–9). Previous studies have suggested that it is not unusual to find healthy individuals who are positive for ANAs (10). Since ANAs are included in the classification criteria for SLE as well as those for autoimmune hepatitis (11,12), analyzing the kinds of variables that affect the levels of ANAs would be helpful for avoiding excessive or deficient classification of these diseases as well as for gaining insight into their etiologies.

Although previous studies showed that ANA positivity was associated with female sex, old age, and being overweight (13,14), genetic components affecting ANA positivity in healthy individuals have never been addressed. Genome-wide association studies (GWAS) have detected many genes that confer susceptibility to connective tissue diseases, including SLE (15–18), and have elucidated the genetic background of biomarkers in general populations (19). Because almost all patients with SLE are positive for ANAs, it is important to confirm that SLE-related genes in the previous GWAS were not merely derived from their associations with ANA positivity.

At present, the number of large-scale studies addressing ANA levels in healthy subjects is quite limited. Detailed analyses of the correlates and genetic components of ANAs in healthy individuals would provide clues to the mechanisms responsible for the production of autoantibodies and the development of autoantibody-mediated autoimmune diseases (20,21). In the present study, we quantified circulating levels of ANAs in 9,575 Japanese volunteers for detailed analyses of the distributions and effects of correlates on ANA production. We also performed a GWAS in 7,148 of the 9,575 subjects to detect susceptibility loci that affect ANA production.

SUBJECTS AND METHODS

This study was approved by the Ethics Committee of Kyoto University Graduate School and Faculty of Medicine.

Study population. This study was performed as a part of the Nagahama Prospective Genome Cohort for Comprehensive Human Bioscience (the Nagahama Study), a community-based prospective multiomics cohort study conducted by the Center for Genomic Medicine at Kyoto University (22). A total of 9,809 volunteers ages 30–75 years in Nagahama City, Shiga Prefecture, Japan were recruited for this study. Written informed consent was obtained from each participant, and all were asked to complete a detailed questionnaire including present and past illnesses and lifestyle.

Exclusion criteria. We excluded volunteers from the association studies if they lacked necessary information or had ever been told that they have or had an autoimmune disease. We also excluded individuals whose answers to the questionnaire suggested that they might have an autoimmune disease. As a result, a total of 9,575 subjects remained for this study. A detailed flow chart of sample exclusion is shown in Supplementary Figure 1 (available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38867/abstract).

Quantification of ANAs and C-reactive protein (CRP). ANAs and CRP in serum samples from volunteers were quantified (23) at SRL, one of the largest clinical laboratory testing companies in Japan. ANAs were quantified by serum dilution using indirect immunofluorescence with HEp-2 cells (TFB). Titers of ANAs with detailed staining patterns (speckled, homogeneous, nucleolar, cytoplasmic, and discrete speckled patterns) were also reported for these subjects. A cutoff level of 1:40 for positivity was applied according to the manufacturer's instructions.

Selection of potential correlates. Age, sex, body weight, smoking, alcohol use, and serum CRP level were selected as potential correlates based on a previous US study (14). CRP was quantified by highly sensitive methods using nephelometry, with a detection limit of 0.051 mg/liter, as previously reported (23).

Statistical analysis of nongenetic studies. The subjects were divided into 2 subgroups based on sex, 9 subgroups based on age (5-year intervals), and 18 subgroups based on sex and age. Associations between ANAs and age and/or sex were assessed by standardized logistic regression analysis. Odds ratios were also calculated with 95% confidence intervals. The associations between ANAs and potential correlates were analyzed by logistic regression analysis, with sex and age as covariates. Statistical analyses were performed using R statistical software (http://www.r-project.org) or SPSS version 18. We set significance levels in a conservative manner using Bonferroni correction for multiple testing.

GWAS. DNA samples from 3,710 of the 9,809 participants in the Nagahama Study were genome-scanned using Illumina HumanHap610, HumanHapOmni2.5-4, or Human HapOmni2.5-8 arrays. A total of 392,801 single-nucleotide polymorphisms (SNPs) that were common between the arrays were selected for the GWAS. We selected 3,185 subjects with call rates of >0.95 who did not show a high degree of kinship (PI_HAT <0.35) and who did not have connective tissue diseases. SNPs that showed P values less than 5×10^{-7} and in Hardy-Weinberg equilibrium ($P > 1 \times 10^{-7}$) with a success rate of >0.95 and a minor allele frequency of >0.05 were selected for a replication study using a TaqMan Assay (Applied Biosystems) with 3,963 of the participants. Population stratification was assessed with genomic control (24). Logistic regression analysis was performed to analyze the genetic influence on the production of ANAs for each SNP, corrected by age and sex. Logistic regression analysis was also used for the conditioning analysis. The associations of the 2 studies were combined using the inverse-variance method. The Jonckheere-Terpstra test was used to assess increasing effects of SNPs on ANA levels in subjects positive for ANAs.

HLA imputation. The HLA–DRB1 locus (the established HLA locus associated with SLE in previous reports) and the HLA–DQB1 locus were imputed using the GWAS data with HLA*IMP:02 (25). The imputation accuracy was evaluated by kappa coefficient with the use of imputation and genotyping data for 589 patients with rheumatoid arthritis and 932 healthy subjects for HLA–DRB1, as previously described

(23), and for 114 patients with thyroid diseases for HLA–DQB1 (Terao: unpublished observations). We analyzed whether each allele of HLA–DRB1 and HLA–DQB1 with imputation accuracy >70% was associated with ANA positivity by logistic regression analysis with additive or dominant models.

Evaluation of linkage disequilibrium (LD). LD between SNPs and HLA–DRB1 alleles was obtained from previous studies (17,26,27). For LD calculation between HLA–DRB1 and HLA–DQB1 alleles, we used genotyping data of 1,000 unrelated healthy Japanese subjects (Terao: unpublished observations).

Evaluation of effects of SLE-related SNPs. A total of 7 SNPs that displayed associations with SLE beyond levels significant in GWAS in a Japanese population (15) and the 5 SNPs in the HLA locus that displayed independent associations with SLE in Europeans (28) were selected to assess their effects on ANA positivity. The associations between these SNPs and ANA positivity were analyzed based on imputation by MaCH (29), using 192 samples in the Nagahama Study genotyped by HumanHapOmni2.5-8, HumanHapOmni2.5s, and HumanExome arrays or using East Asian panels in the 1000 Genomes Project as a reference when they were not directly genotyped.

Statistical analysis of genetic studies. Statistical calculations were performed using Plink software version 1.07 (30) and R statistical software. For all genetic analyses including the GWAS, we set significance levels using the Bonferroni correction for multiple testing.

RESULTS

A total of 9,575 subjects were analyzed for their ANA levels in the current study (Table 1). ANA titers in 45.2%, 12.5%, and 2.8% of the volunteers were ≥1:40, ≥1:80, and ≥1:160, respectively (see Supplementary Table 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38867/abstract). When we analyzed potential correlates of ANA positivity, female sex and old age had higher correlations with ANA positivity, as shown in previous studies (13,14) (corrected P [P_{corr}] $< 1.0 \times 10^{-10}$) (see Supplementary Figure 2 and Supplementary Table 2, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38867/abstract).

When we focused on each staining pattern, 43.7%, 25.3%, 4.7%, 0.9%, and 2.0% of subjects had ANAs with speckled, homogeneous, nucleolar, discrete speckled, and cytoplasmic patterns, respectively, at titers of ≥1:40 (Table 1). The multiple logistic regression analyses revealed that the nucleolar pattern was not associated with age or sex (see Supplementary Table 2 and Supplementary Figure 3, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38867/abstract). Considering the higher frequency of the nucleolar pattern compared with that of the discrete speckled pattern, these results indicated that age and sex do not influence the positivity for each staining pattern in the same manner. Positivity for the speckled pattern was strongly correlated with positivity for all ANAs (see Supplementary Figure 4, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38867/abstract). Associations between other potential correlates and ANAs are shown in Supplementary Table 3 (available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38867/abstract). High CRP levels showed an association with ANA positivity (P_{corr} = 0.0029). We did not find a significant association between obesity and ANA positivity.

Table 1. Characteristics of the subjects in the current study*

	All subjects (n = 9,575)	GWAS (n = 3,185)†	Replication study (n = 3,963)†
Women	66.9	66.0	67.0
Age, mean ± SD years	53.3 ± 13.4	52.0 ± 14.1	53.7 ± 13.5
ANA titer ≥1:40			
All	45.2	48.4	42.5
Speckled	43.7	46.8	41.1
Homogeneous	25.3	29.0	21.3
Nucleolar	4.7	5.1	4.2
Discrete speckled	0.9	0.8	0.9
Cytoplasmic	2.0	1.6	2.3

* Except where indicated otherwise, values are the percent. ANA = antinuclear antibody.
† In the genome-wide association study (GWAS), DNA samples were genome-scanned using Illumina HumanHap610, HumanHapOmni2.5-4, or HumanHapOmni2.5-8 arrays. Genotyping in the replication study was performed using a TaqMan Assay.

Next, we performed a GWAS for ANA positivity. A total of 3,185 participants and 303,506 markers that had passed criteria of inclusion and quality control were used for logistic regression analysis, with age and sex as covariates. As a result, the Q–Q plot indicated an inflation factor of 1.02, suggesting that the current study was free from population stratification (Figure 1). A significant association of rs9405108 in the HLA locus was observed at a P value of 8.9×10^{-8}. Conditioning rs9405108 to detect further associated markers in this region did not result in any markers showing significant associations ($P > 1.0 \times 10^{-4}$) (data not shown). No SNPs in non-HLA regions displayed suggestive associations ($P > 1.0 \times 10^{-5}$). We performed a replication study for rs9405108 using 3,963 participants (Table 1). For technical reasons, SNP rs2395185, which is almost in complete LD with rs9405108 ($D' = 1$ and $r^2 = 0.999$), was genotyped instead of rs9405108. As a result, the

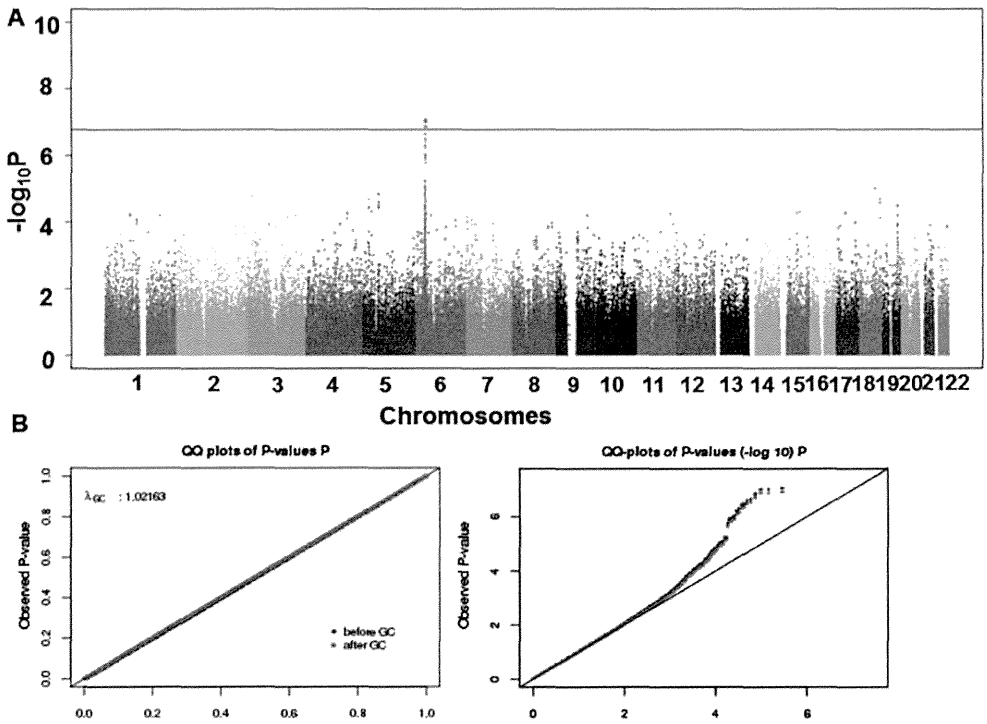

Figure 1. Genome-wide association study (GWAS) results for antinuclear antibody (ANA) production. **A,** Manhattan plot. The horizontal line indicates the significance level of the GWAS based on Bonferroni correction. **B,** Q–Q plots. λ_{gc} = genomic control inflation factor.

association of rs2395185 was replicated (overall $P = 1.3 \times 10^{-11}$) (Table 2).

SNP rs2395185 is located between the *HLA–DRA* and *HLA–DRB5* genes and is in moderate LD with HLA–DRB1*0405 ($r^2 = 0.42$). Considering that major histocompatibility complex proteins are respon-

Table 2. Associations of top SNPs with ANAs or their staining patterns*

SNP	Chr.	Position	ANA staining pattern	Nearest gene	Ref/var	Study	β	SE	OR (95% CI)	*P*
rs2395185	6	32541145	ANA (total)	*HLA–DRA*	G/T	GWAS	0.29	0.055	1.33 (1.20–1.48)	1.4×10^{-7}
						Replication	0.22	0.050	1.24 (1.12–1.37)	1.3×10^{-5}
						Overall	0.25	0.037	1.28 (1.19–1.38)	1.3×10^{-11}
rs2395185	6	32541145	Speckled	*HLA–DRA*	G/T	GWAS	0.29	0.055	1.33 (1.20–1.48)	1.4×10^{-7}
						Replication	0.22	0.050	1.25 (1.13–1.37)	8.3×10^{-6}
						Overall	0.25	0.037	1.29 (1.20–1.38)	7.5×10^{-12}
rs2395185	6	32541145	Homogeneous	*HLA–DRA*	G/T	GWAS	0.31	0.058	1.37 (1.22–1.54)	7.0×10^{-8}
						Replication	0.24	0.058	1.27 (1.13–1.42)	4.6×10^{-5}
						Overall	0.28	0.041	1.32 (1.22–1.43)	2.2×10^{-11}
rs6457300	6	31106721	Nucleolar	*C6orf205*	T/G	GWAS	−0.53	0.12	0.59 (0.46–0.74)	1.2×10^{-5}
						Replication	−0.13	0.11	0.88 (0.70–1.10)	0.26
						Overall	−0.32	0.083	0.73 (0.62–0.86)	0.00013
rs1611185	6	29876323	Discrete speckled	*HLA–G*	T/C	GWAS	1.28	0.29	3.61 (2.03–6.41)	1.2×10^{-5}
						Replication	0.19	0.25	1.21 (0.74–1.99)	0.44
						Overall	0.66	0.19	1.93 (1.32–2.80)	0.00060

* SNP = single-nucleotide polymorphism; ANAs = antinuclear antibodies; Chr. = chromosome; Ref/var = reference allele/variant allele; OR = odds ratio; 95% CI = 95% confidence interval; GWAS = genome-wide association study.

Table 3. Associations of SLE-related SNPs with ANA positivity and SLE susceptibility*

SNP	Chr.	Position	Gene	Ref/var	*P*	ANA OR (95% CI)†	SLE OR (95% CI)‡
Previous loci in Japanese population							
rs10168266	2	191644049	*STAT4*	T/C	0.20	1.08 (0.96–1.2)	1.59 (1.42–1.78)
rs340630	4	88177419	*AFF1*	A/G	0.13	1.08 (0.98–1.2)	1.21 (1.14–1.30)
rs9501626	6	32508322	*HLA*	A/C	0.62	1.04 (0.89–1.22)	1.86 (1.62–2.13)
rs2230926	6	138237759	*TNFAIP3*	G/T	0.15	1.16 (0.95–1.41)	1.75 (1.47–2.08)
rs6964720	7	75018280	*HIP1*	G/A	0.69	0.98 (0.86–1.1)	1.43 (1.27–1.63)
rs2254546	8	11381089	*BLK*	G/A	0.90	1.01 (0.9–1.13)	1.42 (1.25–1.61)
rs6590330	11	127816269	*ETS1*	A/G	0.015	1.14 (1.03–1.27)	1.44 (1.30–1.60)
Independent susceptibility SNPs of HLA locus in European population							
rs9265604	6	31407429	*HLA–B*	C/T	0.78	1.02 (0.92–1.13)	0.83 (0.78–0.89)
rs9378200	6	31680906	*BAT2*	C/T	0.17	0.92 (0.82–1.04)	0.59 (0.52–0.67)
rs9271731	6	32701590	*HLA–DRB1–HLA–DQA1*	G/A	0.41	1.06 (0.92–1.22)	1.34 (1.25–1.45)
rs9469220	6	32766288	*HLA–DQA1*	A/G	0.027	0.88 (0.78–0.98)	0.65 (0.61–0.68)

* SLE = systemic lupus erythematosus (see Table 2 for other definitions).
† For ANA positivity.
‡ For SLE susceptibility.

sible for self recognition and antigen presentation, the association between the polymorphisms in the HLA locus and ANAs seemed reasonable. HLA–DRB1*0405 is associated with a wide range of rheumatic and autoimmune diseases (26,31). This raised the possibility that autoimmune-related markers also had effects on ANA production. We selected SLE as being representative of autoimmune diseases with ANA production, and we analyzed the effects of a total of 7 markers that were reported to be associated with SLE in a previous Japa-

Table 4. Associations of ANA positivity with imputed HLA–DRB1 and HLA–DQB1 alleles*

HLA allele	Model	*P*	Corrected *P*†	OR (95% CI)	Accuracy
HLA–DRB1					
DRB1*0405	Dominant	3.0×10^{-5}	0.00081	1.43 (1.21–1.70)	0.902
DRB1*1302	Additive	3.6×10^{-5}	0.00097	0.69 (0.58–0.82)	0.997
DRB1*1201	Additive	0.00021	0.0057	0.58 (0.44–0.78)	0.704
DRB1*1401	Additive	0.069	1	0.80 (0.62–1.02)	0.746
DRB1*1101	Additive	0.095	1	0.77 (0.57–1.05)	0.827
DRB1*0901	Additive	0.11	1	1.13 (0.97–1.31)	1
DRB1*0701	Additive	0.23	1	0.37 (0.07–1.89)	1
DRB1*0803	Additive	0.33	1	1.10 (0.91–1.33)	0.987
DRB1*1502	Additive	0.52	1	0.95 (0.82–1.11)	0.998
DRB1*0401	Dominant	0.58	1	1.12 (0.74–1.71)	0.883
DRB1*1501	Additive	0.66	1	1.05 (0.86–1.28)	0.992
DRB1*1001	Additive	0.67	1	0.86 (0.42–1.74)	0.909
DRB1*1202	Additive	0.69	1	0.93 (0.64–1.34)	0.964
DRB1*0802	Additive	0.74	1	1.05 (0.77–1.45)	0.808
DRB1*0101	Dominant	0.90	1	1.01 (0.82–1.25)	0.992
HLA–DQB1					
DQB1*0301	Additive	3.5×10^{-5}	0.00095	0.71 (0.61–0.84)	0.888
DQB1*0604	Additive	0.00027	0.0073	0.71 (0.60–0.86)	1
DQB1*0401	Dominant	0.00031	0.0084	1.38 (1.16–1.65)	0.902
DQB1*0302	Dominant	0.0087	0.24	1.30 (1.07–1.59)	1
DQB1*0503	Additive	0.087	1	0.78 (0.58–1.04)	1
DQB1*0303	Additive	0.11	1	1.13 (0.97–1.31)	0.819
DQB1*0201	Dominant	0.15	1	3.46 (0.65–18.39)	1
DQB1*0402	Additive	0.20	1	1.18 (0.92–1.51)	0.907
DQB1*0602	Additive	0.49	1	1.08 (0.87–1.32)	1
DQB1*0601	Dominant	0.67	1	0.97 (0.83–1.12)	1
DQB1*0502	Dominant	0.75	1	0.95 (0.67–1.34)	1
DQB1*0501	Dominant	0.89	1	1.01 (0.83–1.24)	1

* See Table 2 for definitions.
† Corrected by Bonferroni adjustment.

nese study (15). The genotypes of these 7 markers were imputed using subjects in the Nagahama Study genotyped by denser arrays as a reference. All of the alleles showed good quality of imputation ($R^2 > 0.95$), but none of them displayed strong associations with ANA positivity ($P > 0.01$) (Table 3).

Since the HLA locus, especially HLA–DRB1, is the established locus for susceptibility to SLE with multiple independent associations shown beyond ethnicity (15,28,32), we analyzed detailed associations between the HLA locus and ANA positivity. A previous European study identified 5 independent SNPs that confer susceptibility to SLE (28). Because 1 of the 5 SNPs (rs1150703) is monomorphic in Japanese, the results for 4 SNPs are given in the current study (Table 3). None of them showed comparable associations in Europeans. We also performed imputation of HLA–DRB1 and HLA–DQB1 alleles (see Subjects and Methods). While the previous European study suggested the independent association of HLA–DQA1*0102 with SLE, we used HLA–DRB1*1501 and *1302 instead, which explained large parts of the association between HLA–DQA1*0102 and SLE (28). HLA–DRB1*0405, which was moderately tagged by rs2395185, showed a positive association with the smallest P value ($P_{corr} = 0.00081$) (Table 4). HLA–DQB1*0401 also showed a positive association, and HLA–DRB1*1302 and *1201 and HLA–DQB1*0301 and *0604 showed negative associations ($P_{corr} \leq 0.0084$) (Table 4). The associations of HLA–DQB1*0401, *0604, and *0301 seemed to be explained with HLA–DRB1*0405, HLA–DRB1*1302, and a combination of HLA–DRB1*1201 and HLA–DRB1*1101, respectively (r^2 values of 0.99, 0.92, and 0.59, respectively). HLA–DRB1*1501, the strongest susceptibility allele in Japanese (32), did not show a significant association (Table 4).

Considering the negative association of HLA–DRB1*1302 and the lack of association of HLA–DRB1*1501, HLA–DQA1*0102 was assumed to display a suggestive negative association. HLA–DRB1*0901, *0802, and *0401, which showed independent significant positive associations with SLE in Japanese (32), were not associated with ANA positivity.

Next, we addressed the similarities and differences of associations in the HLA locus among ANA staining patterns. Among the 2,820 SNPs in the HLA locus, rs9368726 and rs1964995, both of which were in strong LD with rs2395185 (r^2 values of 1.0 and 0.72, respectively), showed the strongest associations with speckled and homogeneous patterns, respectively ($P = 1.1 \times 10^{-7}$ and $P = 3.6 \times 10^{-8}$, respectively, in the GWAS) (Figure 2A). When we used the genotyping

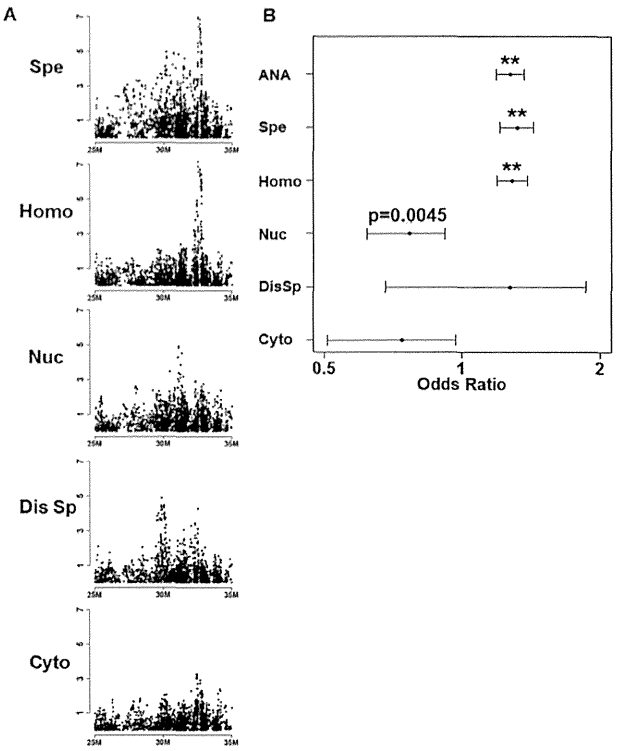

Figure 2. Heterogeneous association of the HLA locus among staining patterns of antinuclear antibodies (ANAs). **A,** Regional Manhattan plots for different staining patterns in the HLA region. **B,** Odds ratios and 95% confidence intervals of associations between rs2395185 and ANAs or their staining patterns. ** = $P < 1.0 \times 10^{-10}$. Spe = speckled; Homo = homogeneous; Nuc = nucleolar; Dis Sp = discrete speckled; Cyto = cytoplasmic.

results of rs2395185 instead of the 2 SNPs, the associations were also observed in the replication study (overall $P = 7.5 \times 10^{-12}$ and overall $P = 2.2 \times 10^{-11}$ for speckled and homogeneous patterns, respectively) (Table 2). The strongest associations with nucleolar and discrete speckled patterns in the HLA locus were observed for rs6457300 and rs1611185, respectively (both $P = 1.2 \times 10^{-5}$) (Table 2). Both SNPs are located >1.4 Mb from rs2395185. The cytoplasmic pattern showed the strongest association with rs9268347 ($P = 0.00052$), which is located 101 kb from rs2395185. We further genotyped rs6457300 and rs1611185 in the replication study, but the associations were not replicated (Table 2).

We focused on rs2395185 since it was the only SNP that demonstrated increasing effects on speckled and homogeneous patterns beyond levels significant in GWAS. Despite its increasing effects on the production of speckled and homogeneous patterns, the SNP displayed a significant decreasing effect on the nucleolar

pattern ($P = 0.0045$) (Figure 2B). Next, we analyzed whether rs2395185 had increasing effects on ANA levels in subjects positive for ANAs. When we examined subjects with ANA titers ≥1:40 and reviewed the staining patterns, the T allele of rs2395185 showed suggestive or significant increasing effects on levels of total, speckled, and homogeneous patterns ($P = 0.12$, $P = 0.016$, and $P = 0.00030$, respectively, by Jonckheere-Terpstra test).

DISCUSSION

The current study provided solid evidence of the distribution and correlates of ANAs in a Japanese adult population. This is the first study to perform GWAS of ANAs in healthy populations and detect a significant locus. The nucleolar pattern has characteristics that differ from those of other staining patterns. Autoantibodies such as anti–U3 RNP, anti-Th/To, or antiribosomal antibodies, associated with systemic sclerosis or SLE, are classified as having the nucleolar pattern of ANAs.

In our study, 12.5% of healthy participants had ANA titers of ≥1:80, which is comparable to previous results in the US (4,754 individuals, 13.8%) (14). The percentages were slightly higher than in previous studies for the cutoff level of 1:40 and comparable for the cutoff level of 1:160 (~26.8–31.7% and ~5.0–8.1%, respectively, in previous studies). Of the 201 subjects who were excluded due to the possibility of having autoimmune diseases, 141 had ANA titers of ≥1:40 (70.1%) (data not shown), suggesting the validity of the exclusion criteria. The increase in ANA positivity in women was confirmed, and this association could partly be explained by sex hormones (33–35). Considering the sex difference in onset of autoimmune diseases, the same undetermined mechanisms related to sex may underlie ANA production in healthy populations.

This study showed a strong effect of age on positivity for ANAs. We did not observe an increase in positivity for ANAs with aging in subjects 30–50 years old ($P = 0.20$) (data not shown); therefore, the elderly populations largely accounted for the association between aging and ANA positivity. The increase in ANAs after age 50 years matches the results in the US study. This association might be explained by dysregulation of immunologic tolerance in the elderly population. Considering the previous reports of high ANA levels in the adolescent population (13,36), the association between ANA positivity and aging in the general population seems to have a "U" pattern (lowest ANA levels at ages with most frequent reproduction). The effects of age and sex on ANAs seemed to differ among the staining patterns. The nucleolar pattern did not display significant associations with age and sex. As discrete speckled patterns showed positive associations, the lack of association of the nucleolar pattern with age and sex cannot be explained by its frequency.

Correlates of ANAs seemed to partly differ between different populations. The current study did not find a significant association between obesity and ANA positivity. However, obesity tended to be inversely related to ANA positivity as in the US study, and the limited number of obese individuals in the current study might explain this nonsignificant association. The association between increased CRP levels and ANA positivity was not found in the previous study. Chronic mild inflammation would lead to the production of ANAs. Since the distribution of CRP levels in subjects differs greatly between the 2 studies, further analysis would clarify the association.

The current study identified rs2395185 in the HLA class II locus as a marker of susceptibility to ANA positivity. It should be noted that a previous study showed an association between rs2395185 and ulcerative colitis (37), suggesting the involvement of rs2395185 with autoimmune processes. Because a previous study showed that the type I interferon (IFN) signature is up-regulated in healthy populations with high ANA titers (38), it will be interesting to analyze the functional roles of rs2395185 in the type I IFN pathway.

The T allele of rs2395185 showed increasing effects on levels of speckled and homogeneous patterns, but a decreasing effect on levels of the nucleolar pattern. This indicates that the nucleolar pattern also differs from the speckled and homogeneous patterns in terms of HLA association. The detailed plots in the HLA locus support the notion of different association patterns among ANA staining patterns. The opposing effect of rs2395185 on levels of the nucleolar pattern indicates that the lack of common association of rs2395185 over staining patterns of ANAs was not due to lower positivity for several staining patterns. As the HLA class II locus is strongly associated with presentation and recognition of antigen, the current results may suggest that ANA production is associated with binding affinity of antigens to the HLA molecule. Since antigens recognized by ANAs contain a wide variety of molecules, the common strong association of 1 polymorphism with speckled and homogeneous patterns suggests similarity or cross-reactivity of antigens that correspond to speckled or homogeneous patterns. The opposing effect also suggests that antigens corresponding to the nucleolar pattern are not presented by common HLA class II alleles with speckled and homogeneous patterns.

As HLA–DRB1*0405 is associated with susceptibility to immunologic disorders or autoantibody production in autoimmune diseases (27,39), the association between ANA production and rs2395185 in LD with HLA–DRB1*0405 might suggest a common mechanism between HLA–DRB1*0405–related autoimmune disease susceptibility and production of ANAs. At the same time, the association raises the possibility that genes conferring susceptibility to ANA positivity might be identified as genes conferring susceptibility to connective tissue diseases.

However, the current study did not detect significant associations between SLE-related SNPs or HLA alleles and ANA positivity. These results indicated that SNPs significantly associated with SLE in the previous study were associated with SLE itself and not with ANAs. Lack of association between ANA production in healthy subjects and rs9501626 or HLA–DRB1*1501, the most significant HLA SNP or HLA–DRB1 allele associated with SLE in the Japanese population, may suggest that autoantigens recognized by ANAs in SLE patients are different from those recognized by ANAs in healthy populations. In fact, a previous study showed that healthy subjects with high ANA titers exhibited an autoantibody profile distinct from that in SLE patients (38). These results may also suggest the involvement of immunologic molecular pathways in SLE development that are not related to ANA production in healthy populations. While we did not find associations of the 7 SNPs in Japanese and the 4 SNPs in Europeans, we observed that 9 of the 11 SNPs had a common direction of association between SLE susceptibility and ANA positivity. All the susceptibility DRB1 alleles in Japanese (HLA–DRB1*1501, *0901, *0802, and *0401) also showed a trend toward increasing ANAs. The common directionality between SLE susceptibility and ANA positivity may be meaningful.

It will be interesting to finely genotype the HLA locus to determine the polymorphisms and mechanisms responsible for causing the associations with ANAs or speckled and homogeneous patterns. None of the polymorphisms display significant associations with nucleolar, discrete speckled, or cytoplasmic patterns. However, considering the low positivity for these staining patterns and the strength of associations in the HLA locus in the current study, increasing the number of subjects would identify yet-to-be-determined polymorphisms associated with these staining patterns. We did not observe significant associations with ANA positivity outside the HLA locus. In addition, none of the polymorphisms outside the HLA locus showed suggestive associations with ANA staining patterns (data not shown). The significance and roles of ANAs in healthy populations have not yet been clarified. Because a previous study showed that the type I IFN signature is up-regulated in healthy populations with high ANA titers (38), it is possible that high ANA titers in healthy populations reflect a pre–autoimmune disease state. Further followup and analyses are necessary to address these points.

Taken together, the current study determined that the HLA class II locus is a locus for susceptibility to ANA production. Genetic overlap between SLE susceptibility and ANA production in healthy populations is limited. The current results indicate that ANAs are not homogeneous autoantibodies with similar characteristics. It is feasible to analyze whether the current results are observed in different populations, especially in Europeans.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Terao had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Analysis and interpretation of data. Terao, Kawaguchi.

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