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.

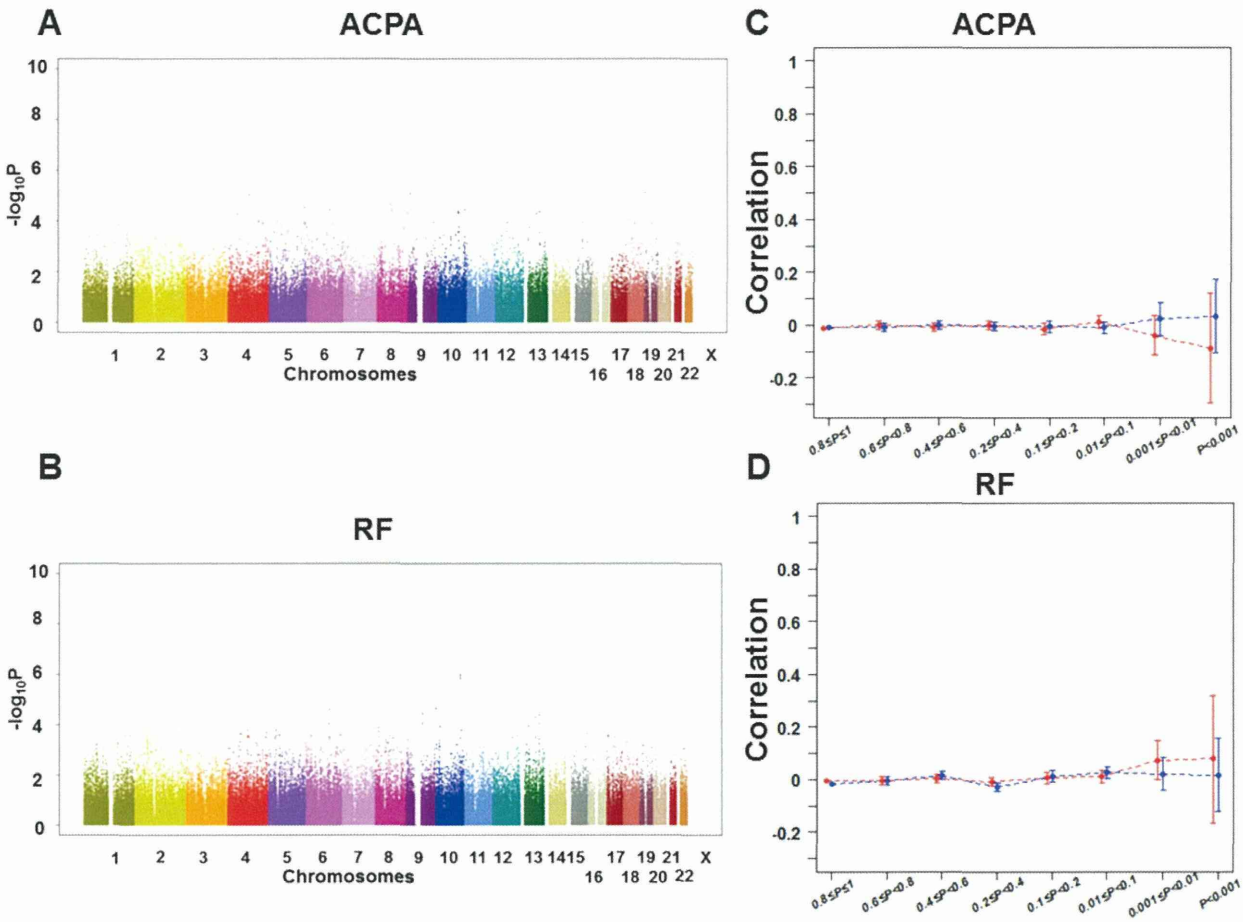

Figure 1. Genetic associations of single-nucleotide polymorphism (SNPs) and anti–citrullinated peptide antibody (ACPA) or rheumatoid factor (RF) positivity. Manhattan plot is shown for positivity of ACPA (**A**) or RF (**B**). No SNPs showed significant associations with positivity of ACPA or RF. Limited 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*

		ACPA			RF		
	No.	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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ORIGINAL ARTICLE

A genome-wide association study of serum levels of prostate-specific antigen in the Japanese population

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ABSTRACT

Background Prostate-specific antigen (PSA) is a useful marker for prostate cancer (PCa) and is widely used for screening of PCa. Previous studies have shown that genetic components influence the levels of PSA, and some of these genetic components would lead to better diagnostic sensitivity and specificity to PCa. However, genetic studies for PSA from Asian countries are limited. Our aim was to identify genetic components influencing PSA levels in the Japanese population using genome-wide association study (GWAS) and to analyse whether genetic components would lead to better screening abilities of PCa.

Methods We performed a GWAS comprising 1086 male subjects using 303 283 single nucleotide proteins, followed by a replication study of 1302 subjects. PSA levels were quantified by chemiluminescence immunoassay method. Quantitative linear regression analysis was performed to assess genetic components of PSA levels. A total of 413 subjects with prostate biopsies were analysed to examine whether genetic determinants would improve diagnostic ability.

Results Rs16856139 in *SLC45A3*, the same region as the previous Chinese study, showed an overall significant association with PSA levels ($p=2.4\times10^{-11}$) along with rs1058205 in *KLK3*. In silico analysis revealed significant association between rs16856139 and expression of *SLC45A3*. Genetic scores of PSA showed a dose-dependent decrease of area under curve (AUC) of PCa and successfully subgrouped the individuals with significantly different AUC ($p\leq0.0097$).

Conclusions Rs16856139, associated with the expression of *SLC45A3*, is significantly associated with the levels of PSA in the Japanese population. Classification of subjects based on PSA genetic determinants would improve screening ability of PSA to detect PCa.

INTRODUCTION

Prostate cancer (PCa) is one of the leading causes of death for male populations in the world, conferring 258 000 deaths a year.[1] Age, ethnicity and genetic components are known to have effects on susceptibility to PCa.[2 3] Among multiple risk factors, old age is strongly associated with increased risk of PCa. African-American population has the highest risk for PCa, while Asians have the lowest.[2] Recent technological developments have enabled us to perform genome-wide association study (GWAS) to detect associated genes or loci with diseases or quantitative trait in an unbiased approach.[4] Previous genetic studies including GWAS have revealed more than 70 susceptibility genes to PCa.[5] Serum levels of prostate-specific antigen (PSA) are widely used for screening of PCa. Although there is a controversy in the usefulness of routine PSA-level quantification in health check-ups of healthy subjects in terms of prolongation of life expectancy,[6] PSA is widely quantified in healthy subjects for the screening of PCa. Prostate hyperplasia, which most male subjects develop when they age, also increases the levels of PSA. Thus, for subjects who are found to have increased levels of PSA, work-up includes prostate biopsy. In Japan, a cut-off level of 4.0 ng/mL for serum level of PSA is applied to select patients for further check-ups.

Because PSA levels are regarded as very important clues to screen for PCa, clarifying correlates of PSA levels other than prostate diseases would lead to improvements in the prediction model of PSA levels and the screening ability of PCa. PSA levels are known to be influenced by genetic components. Around 40–45% of variance of PSA is estimated to be explained by genetic components.[7 8] Previous studies have revealed that *KLK3*, encoding the PSA protein, is the strongest genetic factor to influence the levels of PSA beyond ethnicity.[9 10] The polymorphisms in *KLK3* associated with PSA levels were also shown to be associated with PCa.[9 11] A European GWAS showed that a total of six loci were associated with PSA levels using 16 211 subjects and that four alleles out of the six loci associated with increasing PSA levels were negatively associated with PCa, suggesting personalised cut-off levels of PSA for improvement of efficiency in screening of PCa.[12] Although genetic analyses of PSA levels from Asian countries are limited, a Chinese GWAS recently revealed that three loci, namely, *SLC45A3* at chromosome 1q32.1, *MSMB* at 10q11.23 and *KLK3*, were associated with PSA levels.[13] *MSMB* and *KLK3* are the same loci as found in the European study. These two studies suggest that susceptibility loci overlap between different populations and specific genetic background of PSA levels in different populations. These susceptibility genes should be confirmed in other populations. There might be yet-to-be-identified genetic components associated with PSA levels. Here, we performed a GWAS, followed by a

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replication study, to address genetic components of PSA levels in the Japanese population. We also collected DNA samples from subjects who went through biopsy of prostate and assessed improvement of diagnostic ability for PCa brought about by genetic components.

MATERIALS AND METHODS

Study population

This study was conducted 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 Kyoto University. A total of 9804 volunteers in Nagahama City, Shiga Prefecture, Japan, were recruited in this study from 2008 to 2010.[14] All participants were asked to fill a detailed questionnaire about their present symptoms, past and present illnesses, family history and smoking status. Although a questionnaire of whether the individuals have or had PCa was not included, all participants were asked whether they had cancer and what kind of cancer they had. Based on this questionnaire, we picked up samples with PCa to be excluded.

A total of 293 and 120 subjects who went through biopsy of prostate due to high PSA levels in health check-ups or symptoms suspicious for PCa on rectal digital examinations in the Department of Urology, Aichi Cancer Center Hospital and Kyoto University Hospital, were also recruited for the analysis to assess the usefulness of personalised PSA levels based on genetic components.

Written informed consent was obtained from all participants. This study was approved by the ethics committees of Kyoto University Graduate School of Medicine and Faculty of Medicine and by the ethical committee in the Aichi Cancer Center Hospital.

Measurement of PSA levels

PSA levels were quantified by chemiluminescence immunoassay (CLEIA) method in SRL.inc (Tokyo, Japan). PSA levels for a total of 413 subjects in the Department of Urology, Aichi Cancer Center Hospital and Kyoto University Hospital, were quantified by the CLEIA method at each institution.

Genome-wide association study

GWAS was performed for 3710 participants who joined the Nagahama study during 2008–2009. 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 one of three arrays, namely, Illumina HumanHap610Quad, Omni 2.5–4 and Omni 2.5–8. A total of 394 239 markers that were common across the three arrays were used for the current study. When the same single nucleotide proteins (SNPs) were genotyped for the same subjects by multiple arrays, only consensus genotypes were used. Genotyping quality was controlled by excluding SNPs with call rate below 95%, with minor allele frequency below 5%, and deviating from Hardy–Weinberg equilibrium ($p<5.0\times10^{-6}$). Samples with call rate less than 95%, kinship within this population (PI-hat more than 0.35) and ancestry outliers identified by principal component analysis with HapMap Phase II release 28 dataset as reference[15] were excluded from the analysis. Subjects who reported to be diagnosed PCa or showed PSA levels higher than 10 ng/mL or levels less than the lower limit (0.008 ng/mL) were also excluded. A total of 1086 male subjects were analysed for the quantitative linear regression analysis (QTL) in the current study. Detailed sample filtering is shown in the online supplementary figure S1. QTL was performed by using common log-transformed PSA levels as dependent variables, each SNP as an independent variable and age as a covariate.

Replication and combined studies

An independent set of 1302 male subjects from the Nagahama study showing PSA levels between 0.008 and 10 ng/mL and not reporting to be diagnosed PCa was genotyped by Taqman assay for SNPs showing p value less than 1×10^{-5} in the GWAS. The overall effects of SNPs were evaluated in the combined study using inverse-variance method assuming a fixed effect of each marker.

Imputation analysis

Regional imputation around susceptibility genes or whole genome was conducted by using MACH software[16] with use of HapMap Phase II East Asian panel as reference. SNPs with high imputation quality (Rsq>0.5) were selected for further analyses. LocusZoom was used to draw regional results.[17]

In silico analysis

An association between markers and gene expression was assessed by using Genevar software,[18] which is based on data of gene expression using lymphoblastoid cell lines.[19] Alteration of transcription factor binding motif introduced by markers was evaluated by using HaploReg V.2 software.[20]

Analysis of usefulness of genetic components

We defined the number of risk variants in each subject associated with increasing levels of PSA as a genetic score. Subjects were classified into subgroups according to the genetic scores. PSA levels just before prostate biopsy were analysed to draw receiver operating curves (ROC) to detect PCa and area under the curve (AUC) was calculated in the 413 subjects or subgroups of the subjects. We also applied linear regression analysis in which we set log-transformed PSA levels as a dependent variable and genetic variants associated with PSA levels and/or age as the independent variables. ROC curves were also drawn based on residuals of PSA levels, which were regarded as personally adjusted PSA levels by genotypes (see online supplementary method).

Statistical analysis

Linear regression analyses in GWAS with age as a covariate were performed by PLINK V.1.07.[21] Logistic regression analyses were performed to assess effects on PCa of polymorphisms with significant p values for PSA levels. Other statistical analyses including comparison of AUC were performed using the R statistic system (http://www.R-project.org) or SPSS (V.18). Comparisons of AUC were done by one-tailed DeLong's test. Association between AUC and genetic scores was assessed by Pearson's correlation test. p Values less than 5×10^{-8} and 0.05 were regarded as significant in GWAS and other analyses, respectively.

RESULTS

A GWAS comprising 1086 male subjects was performed with 394 239 markers. After filtering subjects and markers (for detail, see 'Materials and methods' and online supplementary figure S1), 303 283 markers were used for linear regression analysis. Characteristics of the subjects are shown in table 1. As a result, we did not find population structure in quantile–quantile plot ($\lambda=1.02$, figure 1A). We detected a strong association over GWAS significant level in chromosome 1, and the highest peak was found in the *SLC45A3* region ($p=3.7\times10^{-9}$, figure 1B and table 2). We also found one peak near GWAS significant level in

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Table 1 Subjects in the current study

	GWAS	Replication	Biopsy
Number	1086	1302	413
Male ratio	100%	100%	100%
Age	54.3±14.2	57.2±13.0	65.7±6.9
PSA level (median)	0.02–9.57 (0.85)	0.05–9.93 (0.91)	0.1–3892 (8.14)
Prostate cancer	NA	NA	50.3%

Age is expressed by mean±SD.
GWAS, genome-wide association study; NA, not applicable; PSA, prostate-specific antigen.

the *KLK3* region, whose association with PSA has been repeatedly reported beyond ethnicity ($p=5.3\times10^{-7}$, figure 1B and table 2). We additionally detected a total of six signals over three regions with suggestive associations ($2.0\times10^{-7}<p<1.0\times10^{-5}$, table 1), namely, two SNPs in *C9orf93* in chromosome 9, one SNP in *TACC2* in chromosome 10 and three SNPs in *ATP11A* in chromosome 13. None of these eight markers over the five regions displayed deviation from Hardy–Weinberg equilibrium ($p>0.23$). Next we performed a replication study using 1302 subjects by Taqman assay for the eight candidates of susceptibility markers. We selected a total of five markers from each region. Rs10118850 in *C9orf93* region was chosen instead of rs943784, which is in linkage disequilibrium (LD) with rs10118850 and showed the smallest p value ($p=7.0\times10^{-6}$ and 2.2×10^{-6} for rs10118850 and rs943784, respectively, $r^2=0.60$) for technical reasons of designing primers and probe for Taqman assay. The replication results were combined with GWAS results by the inverse-variance method. As a result, rs16856139 in the *SLC45A3* region and rs1058205 in the *KLK3* region demonstrated associations with PSA levels in the replication study ($p=0.00040$ and 1.8×10^{-15}, respectively, table 2). The combined study revealed convincing associations in these loci ($p<2.4\times10^{-11}$, table 2). We did not detect significant associations for the other three SNPs in the replication study (table 2). *SLC45A3* region was recently reported for its association with PSA levels in a Chinese study where rs12409639 was reported as the top marker.[13] Thus, we

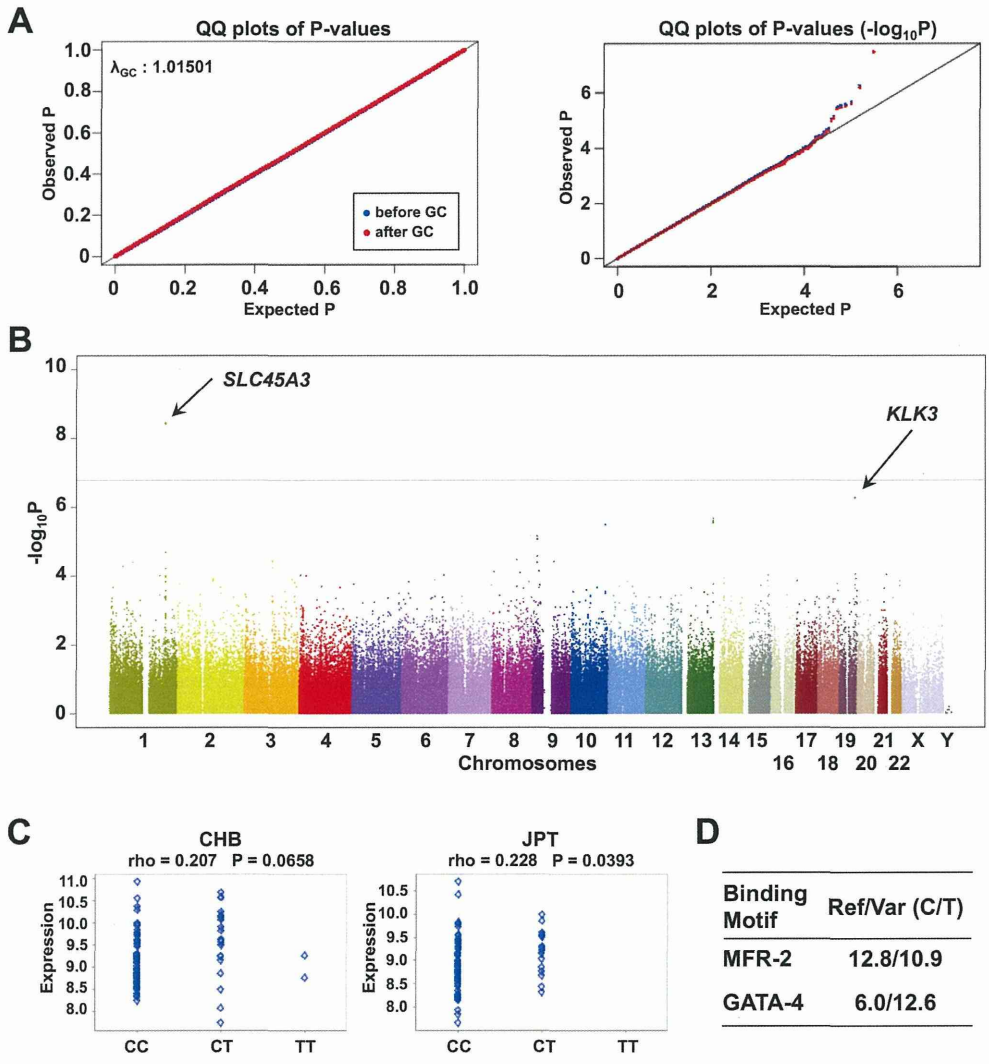

Figure 1 Genome-wide association study (GWAS) results for serum prostate-specific antigen levels in the current study. (A) Quantile–quantile plot and (B) Manhattan plot for GWAS in the current study. (C) In silico analysis between *SLC45A3* expression and rs16856139 using Genevar for the Japanese and Chinese population. The panel is based on output from Genevar. CHB, Chinese population; JPT, Japanese population. (D) In silico analysis for alteration of transcription factor binding introduced by rs16856139. The panel is based on output from HaploRev V.2.

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Table 2 Results of GWAS for PSA in the Japanese population

					GWAS (n=1086)			Replication (n=1302)			Combined (n=2388)		
SNP	Chr	Gene	Ref/var	Position	β	SE	p Value	β	SE	p Value	β	SE	p Value
rs16856139	1	*SLC45A3*	C/T	203 905 087	0.11	0.019	3.7×10^{-9}	0.061	0.017	0.00040	0.084	0.013	2.4×10^{-11}
rs10118850	9	*C9orf93*	C/T	16 061 439	−0.078	0.018	8.6×10^{-6}	0.013	0.015	0.40	−0.027	0.012	0.020
rs7893717	10	*TACC2*	G/A	123 965 713	0.062	0.014	3.2×10^{-6}	−0.022	0.012	0.075	0.016	0.0090	0.070
rs373653	13	*ATP11A*	A/G	112 454 700	0.10	0.021	2.1×10^{-6}	0.00062	0.018	0.97	0.043	0.014	0.0017
rs1058205	19	*KLK3*	C/T	56 055 210	0.069	0.014	5.3×10^{-7}	0.099	0.012	1.8×10^{-15}	0.085	0.0091	8.3×10^{-21}

Chr, chromosome; GWAS, genome-wide association study; PSA, prostate-specific antigen; ref, reference allele; SNP, single nucleotide protein; var, variant allele.

analysed the LD between rs16856139 in the current study and rs12409639 in the Chinese study, and found that both SNPs were in complete LD based on HapMap Phase II data. This means that the two polymorphisms in strong LD with each other were identified from the two studies using different populations. Both of these SNPs are in intron 1 of *SLC45A3* and there were no SNPs in the exon regions of *SLC45A3* in strong LD with these two SNPs ($r^2>0.8$) according to HapMap Project and 1000 Genome Project (figure 2A).[15 22] rs1058205 in *KLK3* was in complete LD with rs2735839, an established marker associated with PSA levels beyond ethnicity (figure 2B).[12 13]

Next, we analysed whether rs16856139 is associated with expression of the *SLC45A3* gene. In silico analysis using Genevar software demonstrated that the T allele of rs16856139, associated with increasing levels of PSA in the current study, was associated with increasing expression of *SLC45A3* (p=0.0066 in the meta-analysis for Chinese (CHB) and Japanese (JPT) populations, figure 1C), suggesting that increasing expression of *SLC45A3* leads to the increasing level of PSA. Further in silico analysis using HaploReg revealed that the T allele introduces alterations in the binding of two transcription factors to this region, namely, GATA4 and MRF-2 (figure 1D). In particular, we found that the T allele was suggested to increase binding capacity of GATA4 to the surrounding region of rs16856139.

Since common associations of *KLK3* and *SLC45A3* between the Japanese and Chinese populations suggest that overlapping genes are associated with levels of PSA beyond ethnicity, we analysed whether variants reported in previous studies were associated with levels of PSA in the Japanese population. When variants in previous studies were not included in the current study, we performed an imputation using data from HapMap Phase II East Asian panel (JPT+CHB) as reference. As a result, we found suggestive associations (p<0.05) for rs10993994 and rs10788160 in the *MSMB* and *BRWD2/FGFR2* regions, respectively (table 3). Rs401681, rs11067228 and rs4430796 in *CLPTM1L*, *TBX3* and *TCF2*, respectively, did not show suggestive associations in this study (table 3).

Because the previous European study revealed some susceptibility markers of PSA levels are associated with PCa and proposed personally adjusted PSA levels considering genetic scores,[12] we performed an association study using a total of 413 subjects from two institutions who went through prostate biopsy due to increased PSA levels or suspicious cancer lesions on digital rectal examination. Previous studies showed that polymorphism in the *KLK3* region is associated with susceptibility of PCa as well as increased level of PSA. In fact, the T allele of rs1058205 showed a suggestive negative association with increasing presence of PCa in the current study (p=0.057, see online supplementary table S1). The T allele of rs16856139 also showed the same negative association tendency with PCa. We drew a ROC based on the presence of PCa and PSA levels to assess the effect of PSA to distinguish PCa. A total of 413 subjects, 217 of whom were diagnosed with PCa, displayed 0.656 of AUC (95% CI 0.603 to 0.709, figure 3A). When subjects were divided into subgroups based on the genetic scores, namely, the number of variants associated with increasing PSA levels, we found that AUC significantly decreased according to the number of variants (p=0.0041, figure 3B, see online supplementary table S2). Subjects with three or more risk variants displayed a significantly lower AUC than subjects without risk variants and significant improvement of AUC in comparison

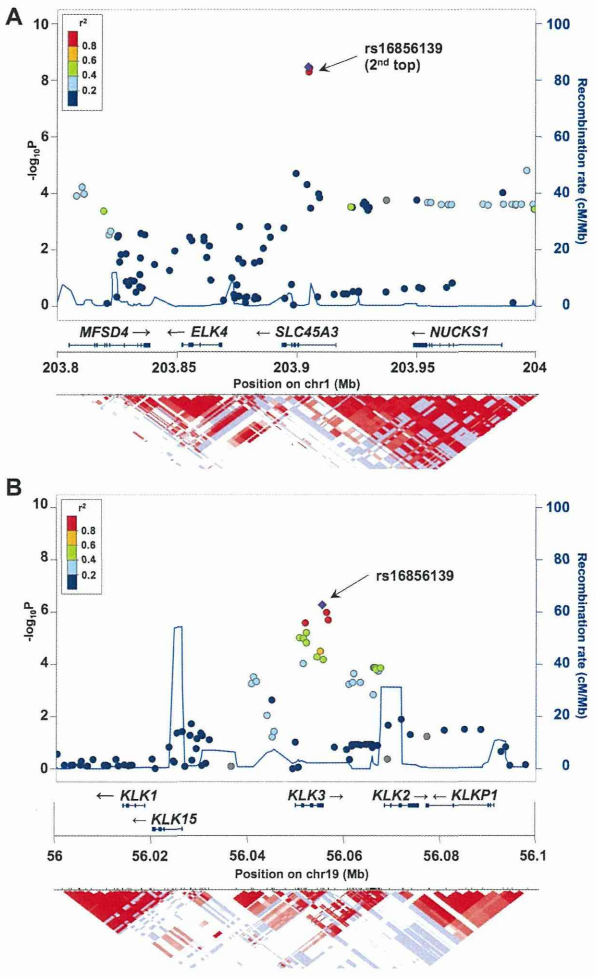

Figure 2 Detailed regional plots in the two regions. Detailed regional plots of p values including results of imputation with HapMap data as reference are indicated for (A) *SLC45A3* and (B) *KLK3*.

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Table 3 Results of SNPs previously reported to be associated with PSA levels

SNP	Chr	Gene	Allele	Position	β	SE	p Value
rs401681	5	*CLPTM1L*	T/C	1 375 087	−0.023	0.014	0.12
rs10993994	10	*MSMB*	T/C	51 219 502	0.033	0.014	0.016
rs10788160	10	*BRWD2/FGFR2*	A/G	123 023 539	0.041	0.017	0.014
rs11067228	12	*TBX3*	G/A	113 578 643	−0.0089	0.014	0.52
rs4430796	17	*TCF2*	G/A	33 172 153	−0.019	0.014	0.17

Chr, chromosome; PSA, -prostate-specific antigen; SNP, single nucleotide protein.

with the unclassified subjects (p=0.0097 and 0.020, respectively). These indicate that genetic scores based on PSA susceptibility alleles successfully group subjects with significantly different AUC among them and lead to improvement of screening ability of PSA. This tendency of dose-dependent decrease of AUC could be observed in both variants (figure 3C,D). When we applied personally adjusted PSA levels by genotypes and draw ROC curve, we did not observe a significant improvement in AUC (p=0.18, AUC=0.663 (95% CI 0.610 to 0.716), see online supplementary figure S2).

DISCUSSION

In the current study, we provided the first evidence of genome-wide QTL for serum PSA levels in the Japanese population. We showed *SLC45A3* and *KLK3* as associated genes with serum PSA level. In addition, we showed that the diagnostic ability of PSA levels for PCa was different among subjects according to the number of variants of *SLC45A3* and *KLK3*. At the same time, we showed that some of the previously reported genes showed suggestive associations in the Japanese population. These results indicate that associated loci partly overlap beyond ethnicity and partly differ. The current results also suggest possibility of personalised cut-off levels of PSA in health check-up based on individual genotypes in the Japanese.

We excluded subjects with PCa from the GWAS and replication studies. In addition, we excluded samples with extremely high PSA levels from the analysis as being suspected to have PCa. Since most of the male subjects develop prostate hyperplasia when they get old, we put age as a covariate to keep the power in the current study instead of excluding subjects with old age. GWAS showed that three other loci displayed suggestive associations with PSA levels, but these associations were not

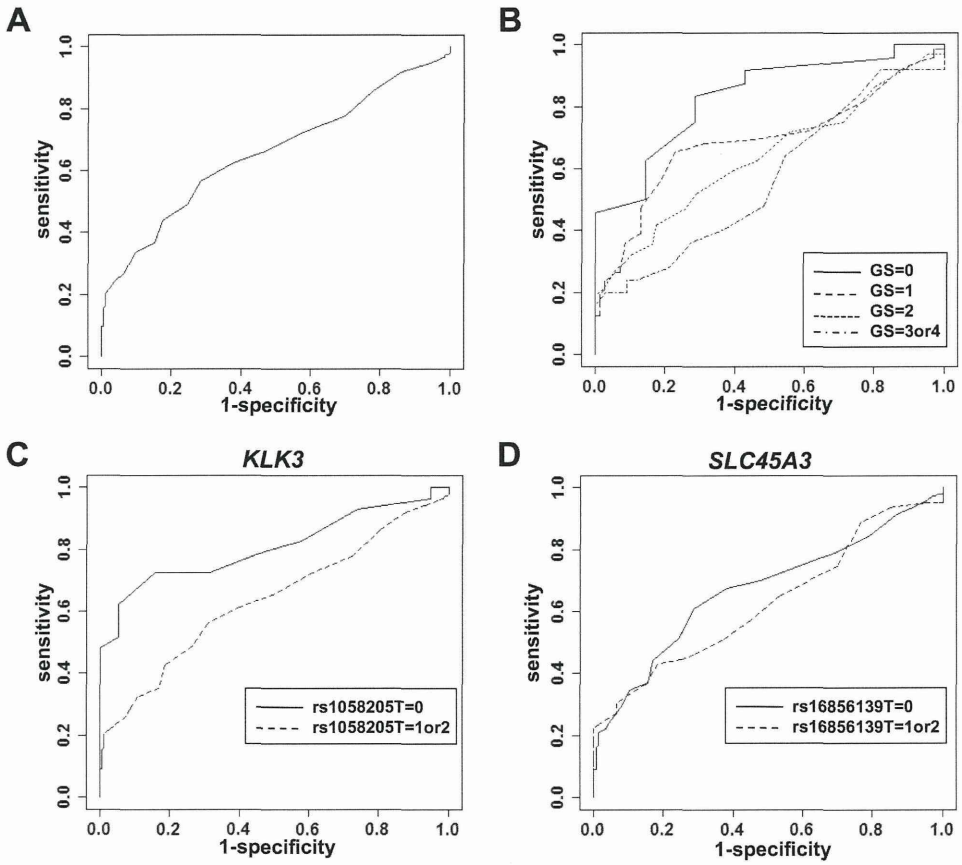

Figure 3 Prostate-specific antigen (PSA) levels demonstrate different area under curve of prostate cancer among the three subgroups based on genetic scores. (A) Receiver operating curve (ROC) curve in all of the 413 subjects is indicated. (B) ROC curves in the four groups with genetic scores of 0, 1, 2 and 3–4 are indicated for the 413 subjects. (C) ROC curves in the two groups with genetic scores in each of the two variants are indicated. The number of the variants associated with increasing PSA levels is defined as a genetic score.

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replicated. Although the current study used approximately 300 000 SNPs spread throughout the genome, denser genotyping would reveal additional loci associated with PSA levels. However, when we applied to markers in HapMap project the imputation method we used in previously reported markers associated with PSA levels, we did not find additional candidate loci for increasing PSA levels (see online supplementary figure S3).

Because both Chinese and Japanese populations independently showed an association between PSA levels and the two SNPs in complete LD with each other, the *SLC45A3* region should have an increasing effect on PSA levels in Asians. Of note, the *SLC45A3* region was not reported as an associated locus with PSA levels in the European population. Lower allele frequencies of rs16856139 and rs12409639 in European populations (2% in HapMap CEU population) may explain the lack of evidence of association between *SLC45A3* and PSA levels. Thus, it will be interesting to investigate whether rs16856139 or rs12409639 show the same association tendency in European populations as Asian populations. SLC45A3 protein, called prostein, was reported to be dominantly expressed in plasma membrane and cytoplasma of prostate.[23] A previous study reported that prostein expression was reactive to androgen.[23] Although the functions of prostein are not fully understood, recent studies have shown deep involvement of prostein in PCa development. Lowered expression of *SLC45A3* in PCa was reported to be associated with shorter PSA-free survival time.[24] Immunohistochemistry for prostein along with PSA was suggested to be useful for diagnosis of PCa and to detect metastatic PCa.[25 26] Thus, while we did not detect a significant association between rs16856139 and PCa in the current study, increasing number of subjects would identify a significant association between them.

In silico analysis revealed that the T allele of rs16856139 associated with increasing PSA levels is also associated with increasing expression of *SLC45A3*. Rs16856139 was not in LD with other polymorphisms in exon regions that alter amino acids of the *SLC45A3* protein. Although we need functional assays to determine whether either rs16856139 or rs12409639 is crucial for regulating expression of *SLC45A3*, the results of the other in silico analysis suggested that rs16856139 induced alterations in the binding of transcription factors and resulted in an increase of *SLC45A3* expression. While the detailed relationship between expression of *SLC45A3* and *KLK3* has not been established, the current results may suggest that rs16856139 indirectly increases PSA levels via increasing *SLC45A3* levels.

Because serum PSA is used for the screening of PCa in many countries including Japan, classification of subjects based on genetic components would improve efficiency of PSA levels for the screening of PCa. This is the first study in the Asian population to report improvements of diagnostic ability of PSA based on genotypes. The current study showed that AUC was the highest in subjects with low genetic scores for PSA (number of risk variants 0) and lowest in those with high genetic scores (number of risk variants 4). This dose-dependent decrease of AUC was contributed by rs1058205 and rs16856139. Since the T alleles of rs1058205 and rs16856139 show increasing effects on PSA levels, subjects with high genetic scores of rs1058205 and rs16856139 should tend to display high levels of PSA. The high PSA levels would make it difficult to distinguish between subjects with high PSA levels due to PCa and an apparent increase due to genetic components in subjects with 3 or 4T alleles of rs1058205 and rs16856139. rs1058205 mainly contributed to the significant difference in AUC between subjects without risk variants and those with three or four risk variants (figure 3C). Considering the comparable effect sizes of these two variants (table 2), rs1058205 may have an effect on PCa susceptibility apart from that on increasing PSA levels. On the contrary, incorporation of personally adjusted PSA levels by genotypes did not reveal a significant improvement of AUC for diagnosis of PCa. This discrepancy should be explained by suggestive protective effects against PCa in alleles of *KLK3* and *SLC45A3*. The current results of dose-dependent change of AUC may suggest that personalised cut-off levels for PSA should be applied based on genotypes to decide whether the subjects should take work-up including prostate biopsy or not. Considering both PSA and prostein are expressed dominantly in prostate tissue and are reported to be associated with PCa, the opposing association of these markers with PSA levels and PCa susceptibility suggests that these polymorphisms are not sole markers associated with PSA levels and that these are possibly related with progression of PCa. Because the number of subjects with prostate biopsy in the current study is limited, further replication studies including studies performed in other Asian countries should be performed to confirm the usefulness of classification of subjects based on genotypes. Adjustment of PSA levels by age did not improve AUC and rather decreased AUC (data not shown), suggesting that aging may have a strong effect on increasing risk of PCa rather than increasing PSA levels.

It will be interesting to perform a meta-analysis of GWAS for PSA levels in Asian populations or in multiethnic populations to increase associated loci with PSA levels and analyse whether these loci are associated with increasing risk of PCa. It would be also feasible to model personalised PSA cut-off level based on genetic components and follow large-scale populations prospectively to evaluate a model to find PCa efficiently and to effectively prolong life expectancy.

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Contributors CT, FM wrote the manuscript. CT analysed the data. NT, KM, KY, NH, NS and OO collected the samples in prostate biopsy. TK, SK, AS, TN and TY collected DNA samples in the Nagahama study. CT, MS and MT performed genotyping. CT, YK, RY and FM designed the study.

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Competing interests None.

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The KSS 2011 Reflects Symptoms, Physical Activities, and Radiographic Grades in a Japanese Population

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Abstract

Background Cultural and ethnic differences are present both in subjective and objective measures of patient health, but scoring systems do not always reflect these differences, and so validation of outcomes tools in different cultural settings is important. Recently, a revised version of The Knee Society Score® (KSS 2011) was developed, but to our knowledge, the degree that this tool evaluates clinical symptoms, physical activities, and radiographic grades in the general Japanese population is not known.

Questions/purposes We therefore asked: (1) how KSS 2011 reflects knee conditions and function in the general Japanese population, in particular evaluating changes with increasing patient age; (2) can objective measures of physical function be correlated with KSS 2011; and (3) does radiographic osteoarthritis (OA) grade correlate with KSS 2011?

Methods Two hundred twenty-six people in the general Japanese population, aged 35 to 92 years, with and without knee arthritis, voluntarily participated in this cross-sectional study. Residents who had no serious disease or symptoms based on a self-assessment were recruited. This study consisted of a questionnaire including self-administered KSS 2011, physical examination, and weightbearing radiographs of the knee. Leg muscle strength, Timed Up and Go test, and body mass index (BMI) were examined in all the participants. Radiographs were graded according to the Kellgren and Lawrence scale (KL grade).

Results Multivariable linear regression analysis showed that KSS 2011 correlated with age (coefficient: -0.30 ± 0.12, $p = 0.011$), BMI (coefficient: -1.47 ± 0.42, $p < 0.001$), leg muscle strength (coefficient: 0.41 ± 0.13, $p = 0.002$), and Timed Up and Go Test (coefficient: -1.96 ± 0.92, $p = 0.034$), but not sex, as independent variables by a stepwise method. KSS 2011 was also correlated with radiographic OA evaluated by KL grade (coefficient: -12.2 ± 2.9, $p < 0.001$).

Conclusions KSS 2011 reflects symptoms, physical activities, and radiographic OA grades of the knee in an age-dependent manner in the general Japanese population.

The Nagahama Prospective Genome Cohort for the Comprehensive Human Bioscience (The Nagahama Study) is composed of the following principal investigators: Fumihiko Matsuda (chairperson), Ryo Yamada, Akihiro Sekine, Shinji Kosugi, and Takeo Nakayama (Kyoto University Graduate School of Medicine and School of Public Health).

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Each author certifies that his or her institution approved the human protocol for this investigation, that all investigations were conducted in conformity with ethical principles of research, and that informed consent for participation in the study was obtained.

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Level of Evidence Level IV, diagnostic study. See Guidelines for Authors for a complete description of levels of evidence.

Introduction

TKA is used widely to relieve pain and improve functional status in patients with symptomatic knee osteoarthritis (OA) [24]. The number of TKAs performed annually has increased in the United States [27] and in other countries [10, 12]. Patient satisfaction is now recognized as an important measure of healthcare quality [4, 13, 17]. However, despite substantial advances in patient selection, surgical technique, and implant design in primary TKA, a study has indicated that 11% to 18% of the patients are still unsatisfied with the operation [5]. In other words, TKA does not perfectly achieve its goal of relieving pain and restoring function in a substantial proportion of patients. One reason is that some patients expect full recovery of the motion of the knee and the ability to participate actively in recreational and physical activities after receiving TKA [18]. To evaluate the reasons why a certain fraction of patients who undergo TKA are dissatisfied, a proper evaluation of patients undergoing TKA is needed. It is also required that the evaluation method be closely related to physical function and, possibly, radiological grade of the patient.

The Knee Society Knee Scoring System® developed in 1989 (KSS 1989) is one of the most often used methods to evaluate patients undergoing TKA [8]. This scoring system has several advantages in terms of its reliability and use, and it has been adopted worldwide [2, 11]. However, increasing importance is being placed on the subjective aspects of evaluation, which have changed from those of prior generations, and were not captured by the KSS 1989. Therefore, in 2011, the new Knee Society Knee Scoring System® (KSS 2011) was refined to better characterize the expectations, satisfaction, and physical activities of more diverse populations of patients who undergo TKA [25]. This new scoring system is based on new scales and validation work [19], and its reliability has been evaluated by our research group and by others [14, 26] with satisfactory results. However, what is an appropriate score in this scoring system in a certain age group remains to be unveiled.

In this study, we asked the following questions: (1) how KSS 2011 reflects knee conditions and function in the general Japanese population, in particular evaluating changes with increasing patient age; (2) can objective measures of physical function be correlated with KSS 2011; and (3) does radiographic OA grade correlate with KSS 2011?

Patients and Methods

We conducted a cross-sectional study of the association between KSS 2011 and clinical symptoms and physical activities in a general Japanese population. Subjects were participants in the Nagahama Prospective Genome Cohort for Comprehensive Human Bioscience (the Nagahama Study) [16]. The Nagahama Study participants were recruited from apparently healthy community residents aged older than 30 years living in Nagahama City, a largely rural city of approximately 124,000 inhabitants in Shiga Prefecture located in the center of Japan. The study has been continuously advertised in the city for residents with no serious disease or symptom based on a self-assessment, and a total of 226 residents with and without knee arthritis voluntarily participated in 2012. We did not specifically exclude patients with knee symptoms or prior knee surgery.

We translated the KSS 2011 questionnaire into Japanese. We used the self-administered questionnaire areas of the KSS 2011 questionnaire, including "symptoms," "patient satisfaction," and "functional activities." The questions on "expectations" were excluded because the participants did not plan to undergo TKA. The area "functional activities" comprises four components: "walking and standing," "standard activities," "advanced activities," and "discretionary activities." The full score of these questions is a maximum of 165 points. We supposed that participants did not have applicable answer choices for some of the questionnaire because the participants may have an impairment involving a body part other than the knee. Thus, in the area of "functional activities," we added a new answer: "I cannot do this because of a problem not related to the knee." Participants who chose this answer were excluded from the analyses. A total of 4% (nine of 224) answered this to the question about "walking and standing," 2.2% (five of 224) for "standard activities," 1.3% (three of 224) for "advanced activities," and 2.7%

Table 1. Baseline characteristics of the participants

Demographic	Mean ± SD
Number of participants	212
Female (ratio)	123 (58%)
Age (years)	60.3 ± 12.2
Height (cm)	161.4 ± 9.1
Weight (kg)	59.4 ± 11.8
BMI (kg/m^2)	22.7 ± 3.3
Leg strength (kg)	26.7 ± 10.3
Up and Go time (seconds)	6.2 ± 1.6

BMI = body mass index; Up and Go = Timed Up and Go Test.

Table 2. Demographic data of subgroups (mean ± SD)

Demographic	30s	40s	50s	60s	70s	80s
Number of participants	17	24	47	73	42	9
Female number (ratio)	12 (70%)	15 (62%)	29 (61%)	48 (65%)	18 (43%)	1 (11%)
Height (cm)	163.4 ± 10.4	163.6 ± 9.9	165.0 ± 8.7	159.3 ± 8.3	159.1 ± 8.8	158.6 ± 7.1
Weight (kg)	60.0 ± 13.3	61.2 ± 13.3	63.4 ± 14.0	56.5 ± 10.7	59.0 ± 8.7	56.1 ± 9.1
BMI (kg/m^2)	22.1 ± 3.6	22.7 ± 3.5	23.0 ± 3.7	22.1 ± 3.3	23.2 ± 2.3	22.2 ± 4.1
Leg strength (kg)	26.4 ± 12.4	28.5 ± 9.8	29.7 ± 12.6	25.4 ± 8.4	26.7 ± 9.6	19.1 ± 6.0
Up and Go time (seconds)	5.5 ± 1.0	5.4 ± 0.9	5.7 ± 0.7	6.2 ± 1.3	6.8 ± 1.5	9.0 ± 4.1
Participants with XP (number)				49	32	6
Radiographic knee OA (number)				19	13	4

BMI = body mass index; Up and Go = Timed Up and Go test; OA = osteoarthritis; XP = Xray photography.

(six of 224) for "discretionary activities." In total, 5.4% (12 of 224) participants were excluded for this reason. The female ratio, average age, and body mass index (BMI) of the 212 participants were 58% (123 of 212), 60.3 ± 12.2 years, and 22.7 ± 3.3 kg/m^2, respectively (Table 1).

Anthropometry measurements included height and weight, which were used to calculate BMI as weight in kg/height in m^2. Quadriceps strength was measured twice on both sides during a 3-second isometric contraction of the knee extensors with a handheld dynamometer (μ-Tas F-1; Anima Co, Chofu, Japan). With the participant in a seated position, the hip and the knee were positioned at 90° angles, and the force sensor was placed 10 cm above the lateral malleolus. The average bilateral maximum muscle strength was used to represent maximum muscle strength. Participants also performed the Timed Up and Go Test [23], which measures the time it takes a participant to stand up from a chair, walk a distance of 3 m, turn, walk back to the chair, and sit down as quickly as possible.

We evaluated weightbearing AP radiographs of both knees, which were performed by experienced radiology technicians. Eighty-eight of the 124 participants who were older than 60 years agreed to the examination. Radiographs of the knee were graded according to the scale described by Kellgren and Lawrence (KL grade) [9]. Two experienced orthopaedists (NT, HI), who were blinded with regard to participant status, read the radiographs in consultation. Knee OA was defined as a KL Grade 2 or higher in either knee. Radiographic knee OA was present in 41% (36 of 87) of the participants who completed the radiographic examination (Table 2). We used the average score of both knees as the variable for analysis.

Simple linear regression analysis was used to identify correlations between the KSS 2011 and age. We divided participants into six subgroups according to age. Because only one participant was older than 90 years, we included this participant in the 80s age group. In the analysis, relationships between the KSS 2011 and physical functions and those between the KSS 2011 and other factors were examined. After excluding weight and height, a stepwise method was applied for multivariable linear regression analysis. The relationship between the KSS 2011 and KL grade was evaluated by simple linear regression analysis separately because of the limited number of participants older than 60 years with radiographic data. All data were analyzed using the statistical package R (http://www.r-project.org/).

Fig. 1 Correlation of KSS 2011 and age is shown. Linear regression analysis showed a significant correlation between KSS 2011 and age.

Results

We found that increased age was correlated with decreasing scores on KSS 2011 (Fig. 1). Because age is an essential factor when deciding a therapeutic strategy, we divided the whole group into six subgroups according to their age. The total scores of the subgroups are 163.5 ± 3.7 in 30s, 158.3 ± 17.1 in 40s, 152.3 ± 18.4 in 50s, 148.0 ± 23.8 in 60s, 152.6 ± 16.1 in 70s, and 127.0 ± 25.8 in 80s (Table 3). The satisfaction component

Table 3. Details of each component in KSS 2011 in subgroups (mean ± SD)

Factor	30s	40s	50s	60s	70s	80s
Symptoms	24.7 ± 0.8	24.2 ± 2.3	22.5 ± 4.1	22.1 ± 4.3	23.3 ± 3.2	19.6 ± 4.8
Patient satisfaction	39.5 ± 1.5	37.9 ± 1.5	34.8 ± 7.0	34.4 ± 7.8	35.5 ± 7.9	31.8 ± 5.6
Walking and standing	30.0 ± 0.0	28.4 ± 5.2	29.1 ± 3.3	27.3 ± 5.5	27.5 ± 6.2	22.0 ± 6.9
Standard activities	29.8 ± 0.7	29.4 ± 1.9	28.4 ± 3.4	27.9 ± 3.8	28.8 ± 2.1	23.7 ± 6.5
Advanced activities	24.7 ± 1.0	24.0 ± 3.4	23.6 ± 2.8	22.7 ± 4.2	23.4 ± 2.6	18.8 ± 5.5
Discretionary activities	14.8 ± 0.7	14.5 ± 1.5	13.9 ± 1.9	13.5 ± 2.5	14.2 ± 2.0	11.2 ± 3.0
Sum of KSS 2011	163.5 ± 3.7	158.3 ± 17.1	152.3 ± 18.4	148.0 ± 23.8	152.6 ± 16.1	127.0 ± 25.8

KSS 2011 = 2011 The Knee Society Score®.

Table 4. Correlation analysis of KSS 2011 and other factors (simple and multivariable linear regression analysis) (mean ± SD)

Factor	Simple		Multivariable	
	Coefficient	p value	Coefficient	p value
Age	−0.45 ± 0.11	< 0.001†	−0.30 ± 0.12	0.011*
Sex	0.74 ± 2.87	0.796	Excluded by a stepwise method	
Height	0.19 ± 0.16	0.218		
Weight	−0.12 ± 0.12	0.309		
BMI	−1.22 ± 0.43	0.005*	−1.47 ± 0.42	< 0.001†
Leg strength	0.42 ± 0.14	0.003*	0.41 ± 0.13	0.002*
Up and Go	−3.42 ± 0.87	< 0.001†	−1.96 ± 0.92	0.034*

* Significant risk ratio ($p < 0.05$); †significant risk ratio ($p < 0.001$); KSS 2011 = 2011 The Knee Society Score®; BMI = body mass index; Up and Go = Timed Up and Go Test.

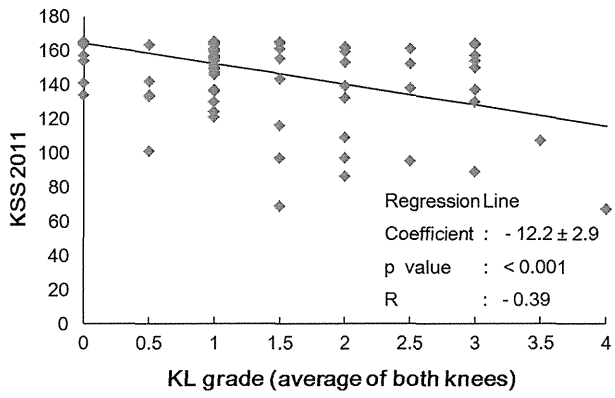

Fig. 2 Correlation between the KSS 2011 and the KL grade is shown. Linear regression analysis showed a significant correlation between KSS 2011 and KL grade.

declined steadily from ages 30s to 50s and was stable from ages 50s to 70s. The scores of people in their 80s were lower than those in the younger age groups for all components.

We found that several measures of physical function and anthropometrics correlated with the score on KSS 2011. Multivariable linear regression analysis showed that KSS 2011 correlated with age (coefficient: −0.30 ± 0.12, $p = 0.011$), BMI (coefficient: −1.47 ± 0.42, $p < 0.001$), leg muscle strength (coefficient: 0.41 ± 0.13, $p = 0.002$), and the Timed Up and Go Test (coefficient: −1.96 ± 0.92, $p = 0.034$), but not sex, as independent variables by a stepwise method (Table 4).

The presence of radiographic arthritis was associated with lower KSS 2011 scores. We found a moderate correlation between the KSS 2011 score and KL grade (Fig. 2). The regression line using the KSS score as an outcome variable (y) and KL grade as a predictor variable (x) was $y = -9.8x + 158.9$ (coefficient: −9.8 ± 2.5, $p < 0.001$, $R = -0.39$).

Discussion

KSS 2011 was designed primarily to evaluate the results of TKA. However, knee function and physical activities vary among patients and are influenced by age and sex, so what should be expected as a desirable score in KSS 2011 after TKA remains unclear. To have a clearer idea about this, some population-derived normative data are important, and it is important that these normative data be determined from relevant national, cultural, and ethnic populations. We found in this study from the general Japanese population, including patients with and without arthritis of the knee, that (1) as age increased, KSS 2011 decreased; (2) objective measures of physical function correlated well with KSS 2011; and (3) the presence of radiographic arthritis was moderately correlated with lower KSS 2011.

This study has several limitations. First, sampling bias certainly exists in many ways in this study. Community residents voluntarily participated in this study and motivated residents would be inclined to participate. Also, residents with a concern or symptoms in their knees may tend to participate. Furthermore, the protocol stipulated

that the radiographic examination was limited to participants older than 60 years, and only two-thirds of these participants agreed to receive this examination. Participants with a higher grade of radiographic OA may have agreed with radiographic examination. Therefore, some sampling bias likely influenced the results. Second, any physical function-related comorbidity may affect the results. Based on this speculation, excluded were patients who had disability unrelated to the knee that could influence patients' scores on KSS 2011; a small percentage (5.4% [12 of 224]) of participants were excluded because they had such disability. Conversely, this also demonstrates that the questionnaire can be answered by most patients without influence from other kinds of disabilities. Third, the component related to "patient expectation" was unavailable in this study because the participants had no plans to receive surgery. However, it is important to point out that patients' expectations about surgery affect their satisfaction with TKA [17], and the results of this study may differ from those in patients who undergo TKA. Fourth, because there is a clear ceiling effect in the KSS 2011, parametric techniques should be cautiously used in statistical analyses. We tested the correlations with a nonparametric analysis and obtained similar results, indicating simple and multiple regression analyses with a general linear model are usable. Also, regression lines by Torbit model are similar to those in the general linear model, supporting the results obtained in this study. Even so, the results obtained here should be handled with the greatest caution. Finally, we used a Japanese version of the questionnaire, but the translated version has not been validated by the cultural adaptation method. Even minor changes in question content can influence patients' estimation of knee pain and disability [21]. A validation study of the translated version is underway.

As expected, KSS 2011 declined with age, which is consistent with other reports [3]. In a previous study, knee function declined gradually with age, and the rate of decrease accelerated in people older than 85 years [18]. Our results are consistent with these previous findings. Collectively, older patients, especially in their 80s, can set much lower goals after TKA compared with younger patients.

It is noteworthy that the KSS 2011 scores correlated with BMI, leg muscle strength, and the Timed Up and Go Test in both the simple and multivariable liner regression analyses (Table 4). These correlations raise several issues. First, greater BMI is associated with knee pain and prevalence of radiographic OA [1, 29] and substantially limits physical activities. This study confirmed that the KSS 2011 score reflects the effects of BMI. Second, a previous study found that lower knee extension strength was associated with knee pain [22, 29]. The strong associations of KSS 2011 with leg muscle strength and the Timed Up and Go Test along with other reports [7, 22] collectively suggest that increased strength can improve the KSS 2011 as well as symptoms and satisfaction. Finally, the simple test of quadriceps strength and the Timed Up and Go Test were well tolerated; these tests are representative tests of knee functions as well as symptoms and satisfaction [29]. These relationships are worth investigating further.

In the current study, 41% (36 of 87) of participants had radiographic knee OA; this percentage agrees with previous reports on the prevalence of OA in the general population [6, 20, 28]. This implies that the participants in this study can be regarded as representative of the general population. The present study showed that KSS 2011 declines with increasing KL grade, which suggests that the severity of radiographic knee OA correlates with knee symptoms and functions and patient satisfaction. However, previous studies showed that the degree of radiographic OA does not correlate strongly with knee pain [15, 20, 29], and symptoms have been more emphasized in therapeutic strategies for OA [24]. Those and this study collectively indicate that KSS 2011 is more suitable than a radiological evaluation when deciding therapeutic strategies.

In summary, this is the first study to our knowledge to apply the KSS 2011 in the general (Japanese) population. The present study has three key findings: (1) In the general population, the KSS 2011 score declined with age. (2) The KSS 2011 score correlated independently not only with age, but also with BMI, the Timed Up and Go Test, and leg muscle strength but not with sex. (3) The KSS 2011 score correlated significantly with KL grade in people older than 60 years. Because TKA is one of the most prevalent operations worldwide, KSS 2011 should be tested in correlation with many aspects of symptoms and functions in a variety of ethnicity, nationality, belonged society, and lifestyle to set an appropriate goal for a patient who undergoes TKA.

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