Table 1. Clinical Characteristics of Study Subjects

Factors	Study Panel (4322)	Replication Panel (5071)
Age, y	53±13	53±13
Sex (men, %)	31.6	32.9
Body height, cm	160.1±8.4	159.9±8.5
Body weight, kg	57.0±10.9	57.3±10.9
Body mass index, kg/m^2	22.1±3.2	22.4±3.3
Waist circumference, cm*	79.8±9.8	80.4±9.3
Medication, %		
Hypertension	15.6	16.1
Hyperglycemia	2.4	2.4
Dyslipidemia	11.5	10.9
Brachial SBP, mm Hg	123±18	124±18
Central SBP, mm Hg	114±19	114±18
DBP, mm Hg	76±11	76±11
PP amplification, mm Hg	9±6	10±6
Radial AIx, %	81.6±13.4	79.8±13.4
Heart rate, bpm	69±10	70±10
baPWV, cm/s	1261±231	1262±227
Type 2 diabetes mellitus	4.0	3.6
Glucose, mg/dL	90±14	90±16
HbA1c, %	5.5±0.5	5.4±0.5
Insulin, μU/mL	5.0±3.1	5.7±6.1
Total cholesterol, mg/dL	207±34	207±35
HDL cholesterol, mg/dL	66±17	65±17
LDL cholesterol, mg/dL	123±31	123±31
Triglyceride, mg/dL	91±58	103±68
FFA, mEq/L	0.69±0.24	0.78±0.31

Values are mean±standard deviation. The study panel consisted of individuals whose fasting blood specimens (>11 h) were available, and the replication panel consisted of individuals whose peripheral blood samples were drawn within 10 h of their last meal. AIx indicates augmentation index; baPWV, brachial-to-ankle pulse wave velocity; DBP, diastolic blood pressure; FFA, free fatty acid; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PP, pulse pressure; and SBP, systolic blood pressure.

*Data available for 4320 (study panel) and 5069 (replication panel) subjects. Type 2 diabetes mellitus was defined as one or more of fasting plasma glucose ≥126 mg/dL, occasional plasma glucose ≥200 mg/dL, HbA1c ≥6.5%, or taking oral antihyperglycemic drugs.

(r=0.190, P<0.001) and total cholesterol levels (r=0.085, P<0.001), and higher brachial SBP (r=0.057, P<0.001) had a significant but weak association with serum FFA levels (Tables S1 and S2 in the online-only Data Supplement). PP amplification markedly increased with FFA quartile (Figure 2), although both brachial and central SBP also showed linear association with FFA levels (Figure S1). In combined analysis with diabetes mellitus status (Figure 2C), differences in PP amplification between the highest (diabetic individuals with highest FFA quartile) and lowest (nondiabetic controls with lowest FFA quartile) subgroups reached ≈4.9 mm Hg. In contrast, AIx exhibited an inverse association with FFA quartile (Figure 2D), whereas baPWV was positively associated with FFA quartile (Figure 2E).

Table 3 summarizes the results of multiple regression analysis for central hemodynamic parameters. Results indicated that serum FFA level was an independent positive determinant for PP amplification (Model 1). Given that FFA was also strongly and inversely associated with AIx, we further adjusted AIx in the regression analysis (Model 2). Although the association between serum FFA and PP amplification remained significant, the regression coefficient of FFA substantially decreased. Lower AIx might, therefore, be involved in the relationship between elevated serum FFA levels and elevated PP amplification. Further, FFA overtook the positive association between plasma insulin level and AIx (Models 3 and 4). Results of these regression analyses indicated that serum FFA levels rather than plasma insulin concentration is a key factor in reducing AIx in subjects with insulin resistance. Waist circumference was not identified as an independent determinant when included instead of body weight in regression Model 4 (P=0.466). The association of FFA with AIx might be independent of adiposity. Serum FFA level also showed an inverse and independent association with central SBP after adjustment for brachial SBP (Model 5).

FFA was a positive determinant for arterial tone when assessed via baPWV (Model 6). However, the associations of FFA with AIx (Model 4), as well as central pressure (Model 1, 2, and 5), were independent of baPWV, suggesting that changes in reflection magnitude rather than transit time of the reflection pressure wave might be involved in the paradoxical relationship between higher FFA and better central hemodynamic profiles.

These findings were supported in the analysis using the replication panel, irrespective of potential differences in fasting status, and no marked sex differences were found in any regression model (Table S3). When MBP was adjusted in the regression models instead of SBP, no marked changes were observed in the regression coefficients of FFA as follows: Model 2 (PP amplification), β=0.030, P<0.001; Model 4 (AIx), β=−0.115, P<0.001; Model 5 (cSBP), β=−0.016, P<0.001; and Model 6 (baPWV), β=0.073, P<0.001. Further, the association of FFA with central hemodynamic parameter was independent of glycemic control levels assessed by hemoglobin A1c: hemoglobin A1c–adjusted regression coefficients of FFA; Model 2, β=0.022, P<0.001; Model 4, β=−0.123, P<0.001; Model 5, β=−0.044, P<0.001; and Model 6, β=0.069, P<0.001.

Discussion

In the present study, we clarified that elevated serum FFA levels were strongly associated with increased PP amplification and decreased AIx, which represents relatively low central BP, in a large-scale general population sample. To our knowledge, this is the first report of a favorable association of FFA with central BP and arterial waveform, which suggests the importance of insulin signaling as a modulator of central hemodynamics. Reduced magnitude of the reflection pressure wave might be involved in this paradoxical relationship.

Insulin resistance and diabetic status have been shown to be favorably associated with AIx and central BP in observational studies in patients with diabetes mellitus[22,23] and general populations,[9,13] as well as in an experimental study using a euglycemic insulin clamp technique.[24] We also reported that not only increased insulin resistance but also reduced insulin sensitivity assessed by an oral glucose tolerance test were factors that

Table 2. Differences in Metabolic Parameters Among the Quartile of PP Amplification (Study Panel)

		Q1	Q2	Q3	Q4	*P*	
Range, mm Hg	Men	<6.5	6.5–9.9	10.0–14.4	≥14.5	Crude	Adjusted
	Women	<4.5	4.5–7.4	7.5–10.9	≥11.0		
No. of subjects		982	1143	1101	1096		
Age, y		57±11	56±12	53±13	47±14	<0.001	
Body height, cm		158.5±7.9	159.0±8.0	160.4±8.1	162.6±8.8	<0.001	
Body weight, kg		55.6±9.7	56.7±10.2	57.0±10.6	58.7±12.4	<0.001	
Brachial SBP, mm Hg		125±19	122±17	122±18	123±19	0.006	
Heart rate, bpm		64±8	67±9	70±10	74±11	<0.001	
Glucose, mg/dL		90±13	90±11	91±16	91±14	0.026	<0.001
Insulin, μU/mL		4.6±2.8	5.0±2.9	5.0±2.9	5.5±3.5	<0.001	<0.001
HOMA-IR		1.04±0.70	1.13±0.74	1.16±0.77	1.26±1.00	<0.001	<0.001
HbA1c, %		5.5±0.4	5.5±0.4	5.5±0.6	5.3±0.6	0.294	0.006
Total cholesterol, mg/dL		210±33	208±33	207±34	202±36	<0.001	0.362
HDL cholesterol, mg/dL		66±17	65±16	66±17	66±17	0.149	0.070
LDL cholesterol, mg/dL		126±30	125±30	123±31	119±32	<0.001	0.022
Triglyceride, mg/dL		91±51	93±57	90±53	90±69	0.618	0.337
FFA, mEq/L		0.63±0.23	0.68±0.23	0.71±0.24	0.74±0.25	<0.001	<0.001

Values are mean±standard deviation. Study subjects were divided into quartile by PP amplification within sex and then combined to avoid potential sex differences. Statistical significance was assessed by analysis of variance (crude model). *P* values adjusted for age, sex, body height, body weight, and use of antihyperglycemic or lipid level–lowering drugs were obtained by linear regression analyses (adjusted model). FFA indicates free fatty acid; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; LDL, low-density lipoprotein; and SBP, systolic blood pressure.

modulate the arterial waveform and reduce central BP.[12] In the present study, however, serum FFA was a more prominent determinant of AIx than insulin. FFA initiates insulin resistance via upstream inhibition of insulin signaling in target cells, whereas increased plasma insulin levels or hyperinsulinemia are secondary responses to compensate for reduced glucose uptake under conditions of insulin resistance. The phase difference in the roles of FFA and insulin may explain the stronger association of FFA with AIx.

In stiffer arteries, the stiffness gradient from aorta to resistant artery was progressively dissipated. Decreased stiffness gradient reduces partial reflection of the forward pressure wave and increases transmission of the pulsatile energy into peripheral microcirculation, which reduces the magnitude of reflection.[14] Chirinos et al[13] recently observed selective stiffening of the aorta, but not more distal arteries, in patients with type 2 diabetes mellitus and suggested that this selective stiffening was the underlying mechanism for the paradoxical observation of a lower reflection magnitude in subjects with type 2 diabetes mellitus. Odaira et al[25] also reported that the contribution of the wave reflection to central hemodynamics might be reduced in subjects with relatively stiff arteries. Because baPWV and AIx were inversely associated with FFA quartile, that is, faster baPWV and lower AIx in higher FFA quartiles, our findings support the pulsatile energy hypothesis.

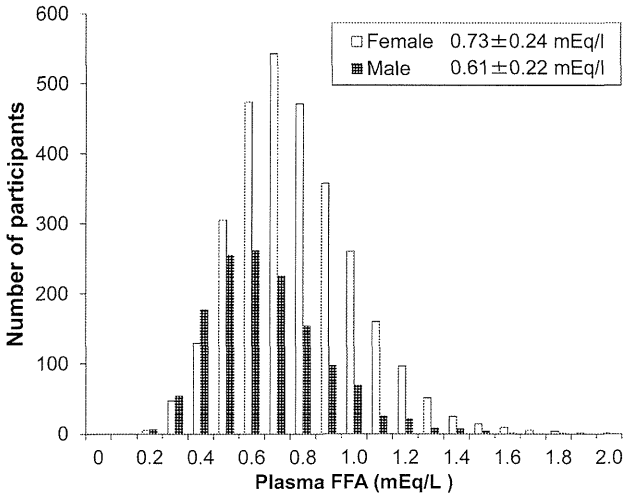

Figure 1. Histogram of serum-free fatty acid (FFA) level (study panel). Serum FFA level was significantly higher in women than in men (analysis of variance, $P<0.001$).

FFA plays a key role in the initiation of insulin resistance by inhibiting glucose uptake of target cells.[15] Given the importance of endothelium-derived nitric oxide in vascular relaxation shown in an animal model of hypertension,[26] the decreased nitric oxide production at the endothelium and subsequent endothelial dysfunction are concomitant mechanisms for the development of insulin resistance via FFA.[15] As our study participants were an apparently healthy general population without severe insulin resistance, reduced endothelial nitric oxide production might be a principal factor in the increased aortic tone and, consequently, larger pulsatile energy in subjects with higher FFA. Insulin increases aortic tone by activating the sympathetic nervous system under the condition of insulin resistance. However, given the weak relationship between serum FFA and insulin levels, the involvement of insulin-mediated sympathetic activation might be independent of the effect of FFA. This is supported by the results of our regression analysis that show the insulin-independent association of FFA with baPWV.

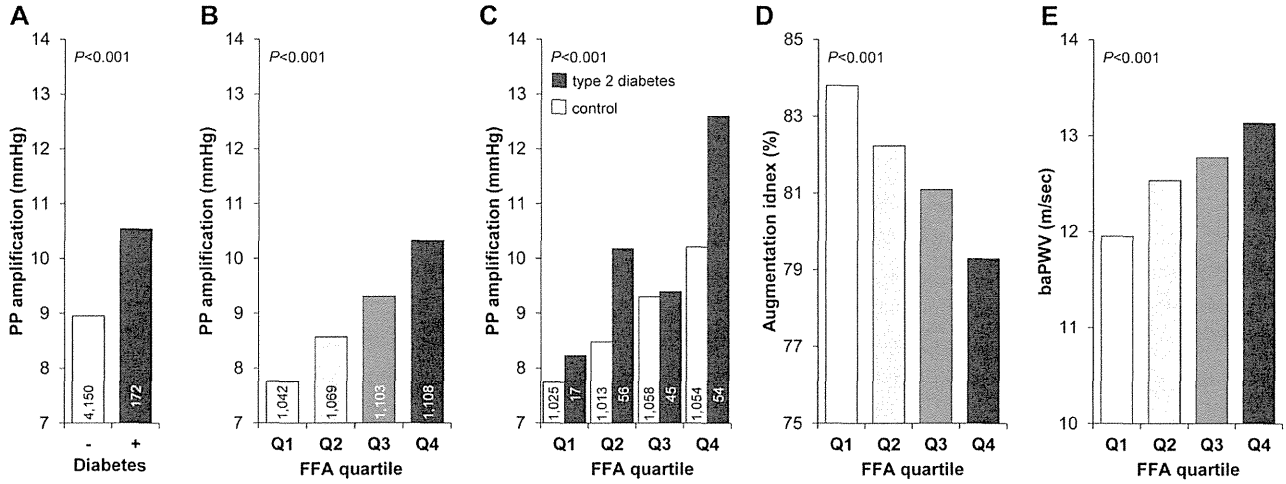

Figure 2. Association of free fatty acid (FFA) quartile with central hemodynamic parameters (study panel). Number of study participants in each subgroup are shown in the column. Statistical significance was assessed by analysis of variance. **A**, **B**, and **C**, pulse pressure (PP) amplification; **D**, augmentation index; **E**, brachia-to-ankle pulse wave velocity (baPWV).

We also investigated associations between plasma lipid parameters and PP amplification, but no remarkable relationships were observed after adjustment for basic covariates. These results further emphasize the importance of serum FFA, but not lipid profile, as a factor involved in central hemodynamics. A previous study in Australia[27] reported that obesity, particularly visceral adiposity, was significantly associated with smaller AIx. As serum FFA is mostly released from enlarged and stressed adipose tissue,[28] FFA might be a confounding factor in the inverse association between visceral adiposity and smaller AIx. No association between waist circumference and AIx was observed in the present study, which supports our hypothesis.

The maximum difference in PP amplification among all FFA quartiles was ≈2.5 mm Hg. This BP difference was somewhat larger than that observed in our previous reports of the association of smoking intensity[29] and insulin sensitivity.[12] The combination of FFA quartile and type 2 diabetes mellitus status further increased the maximum PP difference to 4.9 mm Hg. A previous clinical study, the Conduit Artery Function Evaluation (CAFE) study,[8] clearly indicated that even a 3-mm Hg difference between brachial and central SBP was associated with improved cardiovascular outcomes. Further, several studies have shown that an increase in central SBP of only 1 mm Hg has a substantial effect on large arterial remodeling[30] and silent cerebral damage.[31] Our findings therefore emphasize the importance of measuring serum FFA levels as a potential factor that modulates central hemodynamics and of measuring central BP in epidemiological and clinical settings.

Several limitations to the present study warrant mention. First, we did not directly measure transit time and magnitude of reflection pressure wave. As transit time largely correlate with baPWV, we deduced from results of the regression analysis that reduced magnitude rather than delayed arrival of reflection pressure wave might be involved in the paradoxical relationship between FFA and better central hemodynamic

Table 3. Multiple Linear Regression Analysis for Central Hemodynamic Parameters

Study Panel	Independent Variables	PP Amplification		AIx		Central SBP	baPWV
		Model 1	Model 2	Model 3	Model 4	Model 5	Model 6
Study panel (n=4322)	Type 2 diabetes mellitus	0.027 (0.039)	0.015 (0.007)	−0.014 (0.233)	−0.012 (0.315)	−0.008 (0.039)	0.061 (<0.001)
	Insulin (log-transformed)	0.003 (0.855)	0.019 (0.004)	0.023 (0.100)	0.016 (0.241)	−0.001 (0.855)	0.071 (<0.001)
	AIx, %		−1.004 (<0.001)				
	FFA, mEq/L	0.146 (<0.001)	0.022 (<0.001)		−0.123 (<0.001)	−0.044 (<0.001)	0.069 (<0.001)
Replication panel (n=5071)	Type 2 diabetes mellitus	0.038 (0.001)	0.020 (<0.001)	−0.016 (0.130)	−0.018 (0.082)	−0.012 (0.001)	0.055 (<0.001)
	Insulin (log-transformed)	0.028 (0.043)	0.037 (<0.001)	0.049 (<0.001)	0.009 (0.497)	−0.009 (0.043)	0.082 (<0.001)
	AIx (%)		−1.003 (<0.001)				
	FFA (mEq/L)	0.138 (<0.001)	0.023 (<0.001)		−0.115 (<0.001)	−0.045 (<0.001)	0.051 (<0.001)

Values are standardized regression coefficients (β). *P* values are shown in parenthesis. Adjusted factors were as follows: age, sex, body height, body weight, taking medication for hypertension or dyslipidemia, SBP, heart rate, total cholesterol, and baPWV. In regression Model 6, heart rate and baPWV were not adjusted. AIx indicates augmentation index; baPWV, brachial-to-ankle pulse wave velocity; FFA, free fatty acid; PP, pulse pressure; and SBP, systolic blood pressure.

profiles. More detailed waveform analysis would be needed to obtain conclusive evidence. Second, as this was a cross-sectional study, a longitudinal study is required to confirm the prognostic significance of central SBP differences arising from differences in serum FFA levels. Third, no information on the class of antihypertensive drugs was available for the Nagahama cohort sample, though β-blockers and vasodilators have substantial class effects on central BP that are well documented.[4–8] Given that the associations of FFA quartile with AIx and PPa were independent of antihypertensive medication, our results might be nondifferential and independent of the class effects of antihypertensive drugs.

Perspectives

In conclusion, we found that serum FFA level is an important factor influencing central hemodynamics. Our results might help identify the as yet unidentified mechanisms behind the favorable effects of insulin resistance and type 2 diabetes mellitus on the central hemodynamic profile.

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Disclosures

The authors have no conflicts of interest to disclose.

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Novelty and Significance

What Is New?

- Elevated serum–free fatty acid (FFA) levels were strongly associated with reduced magnitude of arterial reflection pressure wave and relatively low central blood pressure.
- Central pressure differs by ≈4.9 mm Hg because of serum FFA levels and diabetic status.

What Is Relevant?

- Insulin resistance and diabetic status have been shown to be favorably associated with arterial waveform and central blood pressure.

Serum FFA was a more prominent determinant of arterial waveform than insulin.

Summary

Serum FFA was a factor that was favorably associated with central hemodynamics. A favorable association of FFA with central BP and arterial waveform suggests the importance of insulin signaling as a modulator of central hemodynamics.

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

The Nagahama Study

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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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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

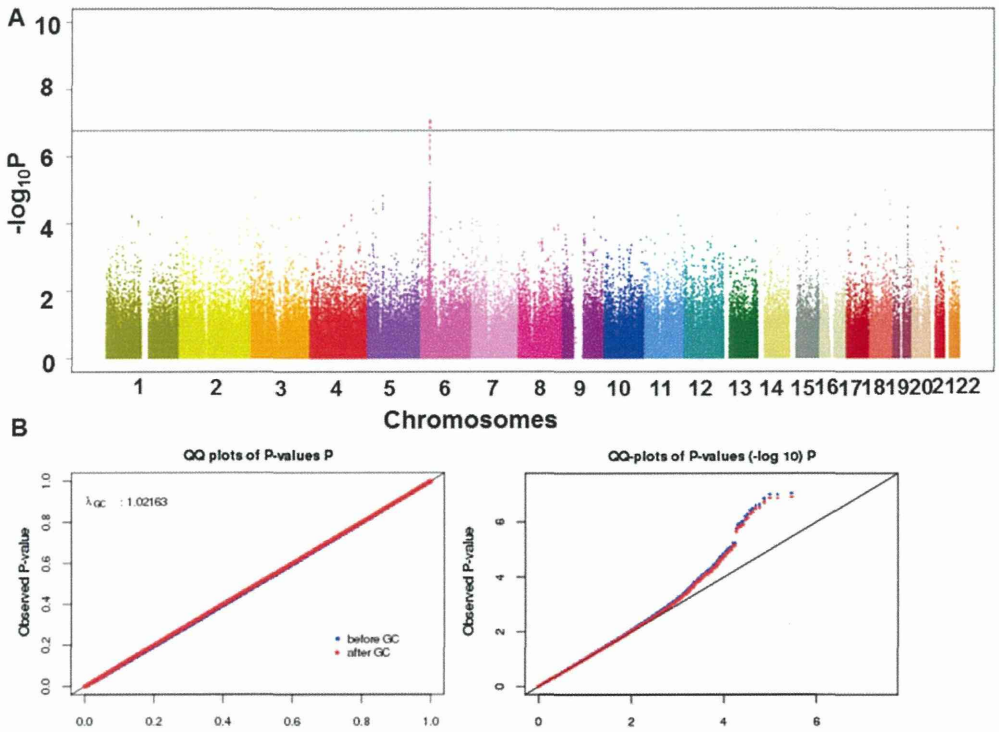

Figure 1. Genome-wide association study (GWAS) results for antinuclear antibody (ANA) production. **A,** Manhattan plot. The horizontal line indicates the significance level of the GWAS based on Bonferroni correction. **B,** Q–Q plots. λ_{gc} = genomic control inflation factor.

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

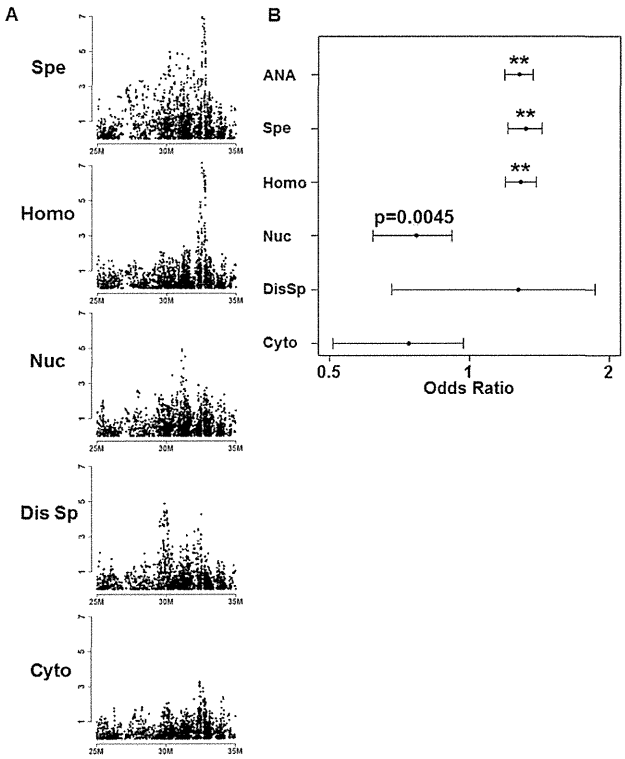

Figure 2. Heterogeneous association of the HLA locus among staining patterns of antinuclear antibodies (ANAs). **A,** Regional Manhattan plots for different staining patterns in the HLA region. **B,** Odds ratios and 95% confidence intervals of associations between rs2395185 and ANAs or their staining 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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Identification of three novel genetic variations associated with electrocardiographic traits (QRS duration and PR interval) in East Asians

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The electrocardiogram has several advantages in detecting cardiac arrhythmia—it is readily available, non-invasive and cost-efficient. Recent genome-wide association studies have identified single-nucleotide polymorphisms that are associated with electrocardiogram measures. We performed a genome-wide association study using Korea Association Resource data for the discovery phase (Phase 1, *n* = 6805) and two consecutive replication studies in Japanese populations (Phase 2, *n* = 2285; Phase 3, *n* = 5010) for QRS duration and PR interval. Three novel loci were identified: rs2483280 (*PRDM16* locus) and rs335206 (*PRDM6* locus) were associated with QRS duration, and rs17026156 (*SLC8A1* locus) correlated with PR interval. *PRDM16* was recently identified as a causative gene of left ventricular non-compaction and dilated cardiomyopathy in 1p36 deletion syndrome, which is characterized by heart failure, arrhythmia and sudden cardiac death. Thus, our finding that a *PRDM16* SNP is linked to QRS duration strongly implicates *PRDM16* in cardiac function. In addition, C allele of rs17026156 increases PR interval (beta ± SE, 2.39 ± 0.40 ms) and exists far more frequently in East Asians (0.46) than in Europeans and Africans (0.05 and 0.08, respectively).

INTRODUCTION

The electrocardiogram (ECG) has several advantages in detecting cardiac diseases; it is readily available, noninvasive and cost-efficient. QRS complex represents ventricular depolarization, and QRS duration represents the conduction time from atrioventricular node (AV node) to His-Purkinje system and ventricular myocardium (1). PR interval is the time between the onset of atrial depolarization (P-wave) and the onset of ventricular depolarization (R-wave).

QRS duration and PR interval are believed to reflect patient outcomes in several heart diseases (2–4). A diseased ventricular conduction system can lead to life-threatening bradyarrhythmias and tachyarrhythmias (5). Longer QRS duration is a predictor of mortality and sudden death in the general population (6) and in those with hypertension and coronary artery disease (7).

ECG measurements are believed to be complex traits with multiple genetic and environmental determinants (8). The heritability of each ECG measurement ranges from 30 to 50% in

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several ethnic groups (8–13). Recent genome-wide association studies (GWASs) have identified single-nucleotide polymorphisms (SNPs) that are associated with PR interval (14,15), QRS duration (14,16) and QT interval (17,18). In particular, QT interval has been studied extensively in European descendants by the QTGEN (17) and QTSCD (18) consortiums, and we have also reported a GWAS in East Asians (19). With regard to PR interval, two GWASs reported eight loci in European descendants, five of which (*SCN5A-SCN10A*, *NKK2–5*, *CAV1/CAV2*, *SOX5* and *TBX5*) were linked to atrial fibrillation (AF) (14,15). Twenty-two loci were correlated with QRS duration in two GWASs of European descendants (14,16), and greater number of risk alleles for prolonged QRS duration was also associated with the risk of ventricular conduction defects (16).

No GWAS on PR interval or QRS duration has been performed in the Asian population. To determine the genetic architecture of PR interval and QRS duration in Asians, we conducted a GWAS using Korea Association Resource (KARE) data during the discovery phase (Phase1, $n = 6805$) and two consecutive replication studies in Japanese populations (Phase 2, $n = 2285$; Phase 3, $n = 5010$) (Supplementary Material, Fig. S1).

RESULTS

Discovery GWASs

The clinical characteristics of subjects in the discovery GWAS and two replication studies are described in Table 1. The genomes of discovery subjects were scanned to identify genetic variations that were associated with QRS duration and PR interval. The genotypes in this study consisted of experimentally genotyped SNPs and computationally imputed SNPs. In total, 2.1 million SNPs were examined in the linear regression model as independent variables of ECG traits, controlling for age, sex, recruitment area, body mass index, systolic blood pressure and height as covariates. Q–Q plots of the GWAS in Koreans are shown in Supplementary Material, Figure S2.

All *P*-values are charted in Figure 1A (QRS duration) and B (PR interval), plotting $-\log_{10}(p)$ against the chromosomal position on Manhattan plots and are shown in Supplementary Material, Tables S1 and S2. The red line in Figure 1 indicates a genome-wide significance level ($P < 5 \times 10^{-8}$), and the blue line indicates a suggestive level ($P < 1 \times 10^{-4}$). Two loci (*CDKN1A* and *SETBP1*) for QRS duration and three loci (*SLC8A1*, *SCN5A/SCN10A* and *CAV1/CAV2*) for PR interval met the genome-wide significance level in the discovery phase. The 323 SNPs for QRS duration and 341 SNPs for PR intervals had *P*-values that were lower than the suggestive level, encompassed by 21 and 20 loci, respectively. The lead SNP for each locus is listed in Supplementary Material, Table S3.

The *SETBP1*, *CDKN1A*, *SCN5A* and *HAND1* regions for QRS duration and *CAV1*, *SCN10A* and *TBX5* regions for PR interval have previously been reported (14–16). The remaining suggestive loci shown in Supplementary Material, Table S3 were examined in replication studies of Japanese populations.

Table 1. Clinical characteristics of subjects in each phase

Variables	Phase 1 KARE	Phase 2 Japanese	Phase 3 Japanese
n (% male)	6805 (50.4%)	2285 (31.9%)	5010 (33.3%)
Age, years	51.6 (8.7)	49.8 (13.9)	56.7 (13.4)
BMI, kg/m^2	24.6 (3.1)	22.2 (3.2)	22.6 (3.2)
SBP, mm Hg	116.4 (17.9)	120.1 (16.8)	126.1 (19.1)
Height, cm	160.6 (8.7)	160.4 (8.3)	158.6 (8.7)
PR interval, ms	163.2 (35.9)	158.1 (21.4)	158.7 (22.0)
QRS duration, ms	90.3 (10.3)	97.0 (8.2)	95.5 (9.0)

Data are presented as mean (standard deviation).
BMI, body mass index; SBP, systolic blood pressure.

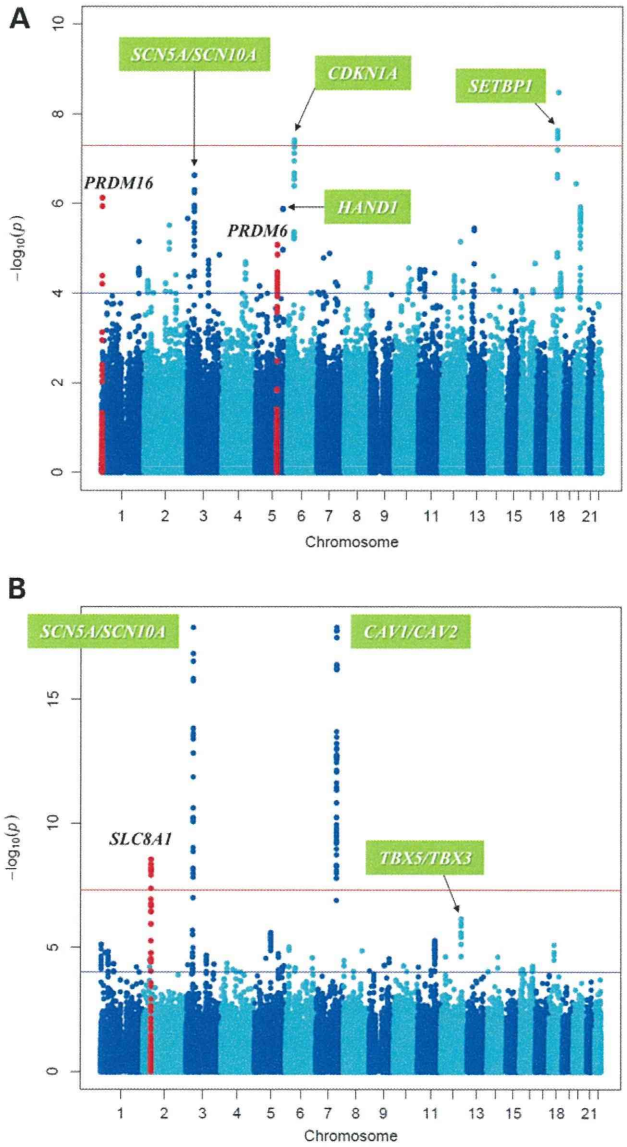

Figure 1. Manhattan plot of genome-wide association signals from Phase 1 Study. $-\log10(p)$ values are plotted against chromosomal base-pair positions. Green label indicates previously reported loci for QRS duration and PR interval, and red dots indicate previously unreported loci showing associations with QRS and PR in the Phase 1 study and tested for replication. The red line represents the genome-wide significance level ($P = 5 \times 10^{-8}$), and the blue line represents a *P*-value of 1×10^{-4}. (**A**) QRS duration and (**B**) PR interval.

Novel genetic variant of QRS duration and PR interval in East Asians

Seventeen suggestive loci for QRS duration and 17 suggestive loci for PR interval were identified in the discovery GWAS. To confirm these findings, two consecutive replication tests were conducted in two independent Japanese populations.

We carried out Phase 2 follow-up *in silico* genotyping for 34 SNPs in 2285 Japanese individuals (Supplementary Material, Table S4). In the meta-analysis of Phases 1 and 2, two SNPs (rs2483280 and rs17026156) reached genome-wide significance *P*-value. These two SNPs and five additional SNPs that became better *P*-values than Phase 1 in meta-analysis of Phase 1 + 2 were genotyped in a subsequent de novo replication study in 5010 Japanese population (Phase 3). Three SNPs (rs2483280 and rs335206 for QRS duration and rs17026156 for PR interval) reached genome-wide significance in the meta-analyses of Phases 1, 2 and 3 (Table 2).

The genetic regions of these three SNPs and their association results are depicted as signal plots in Figure 2. rs2483280 lies in the third intron of *PRDM16* (based on the NM_022114.3 transcript), rs335206 resides in the fifth intron of *PRDM6* (based on the NM_001136239.1 transcript) and rs17026156 is located 21 kb upstream of *SLC8A1*.

Extension of variants identified in European descendants to Koreans

To compare the genetic architecture of QRS duration and PR interval between Europeans and Koreans, the SNPs that were previously identified in European descendants were examined in discovery GWAS (Table 3 and Supplementary Material, Table S5). Three GWASs reported 21 SNPs for QRS duration and 10 SNPs for PR intervals (14–16). In the KARE genotype data of discovery GWAS, there were only five SNPs that matched the reported SNPs. Thus, we added SNPs with linkage disequilibrium (LD) ($r^2 > 0.8$ and $D' > 0.9$) of the lead SNPs in the European studies. A total of 22 SNPs were examined for their association in Koreans (Supplementary Material, Table S5).

Seven of 14 QRS-related loci, 3 of 5 PR-related loci and all 3 loci for both traits were associated with Koreans, based on $P < 0.05$ (Fig. 3). Of the seven QRS duration-associated loci, three (*HAND1-SAP30L*, *CDKN1A* and *SETBP1*) had *P*-values of $<1 \times 10^{-5}$. The three loci (*EXOG-SCN5A-SCN10A*, *CAV1-CAV2* and *TBX5-TBX3*) that were linked to both traits were also significantly associated with Koreans. Further, the *EXOG-SCN5A-SCN10A* and *CAV1-CAV2* regions had large effect sizes compared with other loci (beta ± SE = 4.33 ± 0.56, $P = 1.45 \times 10^{-14}$ and beta ± SE = 3.21 ± 0.42, $P = 3.33 \times 10^{-14}$, respectively).

DISCUSSION

In silico annotation of novel SNP sites

Our discovery GWAS in Koreans and two replication studies in Japanese identified three novel loci for QRS duration and PR interval: rs2483280 (*PRDM16* locus) and rs335206 (*PRDM6* locus) for QRS duration and rs17026156 (*SLC8A1* locus) for PR interval. Because the three SNPs lay in noncoding regions, we examined their function in regulating gene expression

Table 2. Replication results of novel SNPs in each phase and meta-analysis

SNP ID	CHR	BP	Gene[a]	Coded allele	Phase 1 ($n = 6805$)			Phase 2 ($n = 2285$)			Meta-analysis (Phase 1 + 2)			
					AF	Beta ± SE	*P*-value	AF	Beta ± SE	*P*-value	Beta	*P*-value	*Q*	I^2
QRS														
rs2483280	1	3 245 399	*PRDM16*	A	0.26	−0.91 ± 0.18	7.47×10^{-7}	0.28	−0.61 ± 0.24	0.010	−0.80	$\mathbf{3.83 \times 10^{-8}}$	0.33	0.00
rs335206	5	122 532 465	*PRDM6*	T	0.33	−0.76 ± 0.17	8.38×10^{-6}	0.34	−0.50 ± 0.23	0.026	−0.67	9.63×10^{-7}	0.37	0.00
PR														
rs17026156	2	40 614 469	*SLC8A1*	C	0.39	2.39 ± 0.40	$\mathbf{2.85 \times 10^{-9}}$	0.28	2.00 ± 0.68	0.003	2.29	$\mathbf{3.79 \times 10^{-11}}$	0.62	0.00
rs7948943	11	97 642 455	Intergenic	T	0.29	1.96 ± 0.43	5.21×10^{-6}	0.28	1.00 ± 0.68	0.139	1.68	3.45×10^{-6}	0.23	30.47
rs3136189	16	13 942 202	*ERCC4*	C	0.24	1.82 ± 0.46	7.60×10^{-5}	0.24	0.96 ± 0.71	0.176	1.56	4.97×10^{-5}	0.31	3.86
rs6499591	16	71 464 505	*ZFHX3*	C	0.21	−1.97 ± 0.49	5.58×10^{-5}	0.24	−1.12 ± 0.70	0.108	−1.69	2.39×10^{-5}	0.32	0.00
rs17744182	18	28 286 523	*GAREM*	A	0.39	1.84 ± 0.41	7.91×10^{-6}	0.39	0.88 ± 0.62	0.157	1.55	6.51×10^{-6}	0.20	39.97
					Phase 3 ($n = 5010$)			Meta-analysis (Phase 1 + 2 + 3)						
					AF	Beta ± SE	*P*-value	Beta	*P*-value	*Q*	I^2			
QRS														
rs2483280	1	3 245 399	*PRDM16*	A	0.28	−0.68 ± 0.17	8.19×10^{-5}	−0.75	$\mathbf{1.51 \times 10^{-11}}$	0.55	0.00			
rs335206	5	122 532 465	*PRDM6*	T	0.35	−0.64 ± 0.16	8.06×10^{-5}	−0.66	$\mathbf{3.19 \times 10^{-10}}$	0.66	0.00			
PR														
rs17026156	2	40 614 469	*SLC8A1*	C	0.29	1.74 ± 0.45	9.44×10^{-5}	2.08	$\mathbf{2.58 \times 10^{-14}}$	0.55	0.00			
rs7948943	11	97 642 455	Intergenic	T	0.29	−1.18 ± 0.45	9.12×10^{-3}	0.56	4.16×10^{-2}	0.00	92.25			
rs3136189	16	13 942 202	*ERCC4*	C	0.24	−0.82 ± 0.47	8.35×10^{-2}	0.61	4.02×10^{-2}	0.00	87.72			
rs6499591	16	71 464 505	*ZFHX3*	C	0.24	−0.11 ± 0.47	8.10×10^{-1}	−1.03	7.25×10^{-4}	0.02	73.24			
rs17744182	18	28 286 523	*GAREM*	A	0.40	1.26 ± 0.42	2.40×10^{-3}	1.43	6.25×10^{-8}	0.38	0.00			

[a]Genes are defined as the gene closest to the SNP within a 200-kb window (HaploReg v2). Bold indicates genome-wide significant *P*-values (5×10^{-8}).
CHR, chromosome; BP, base pair; AF, coded allele frequency; *Q*, *P*-value for Cochrane's Q statistic; I^2, heterogeneity index.

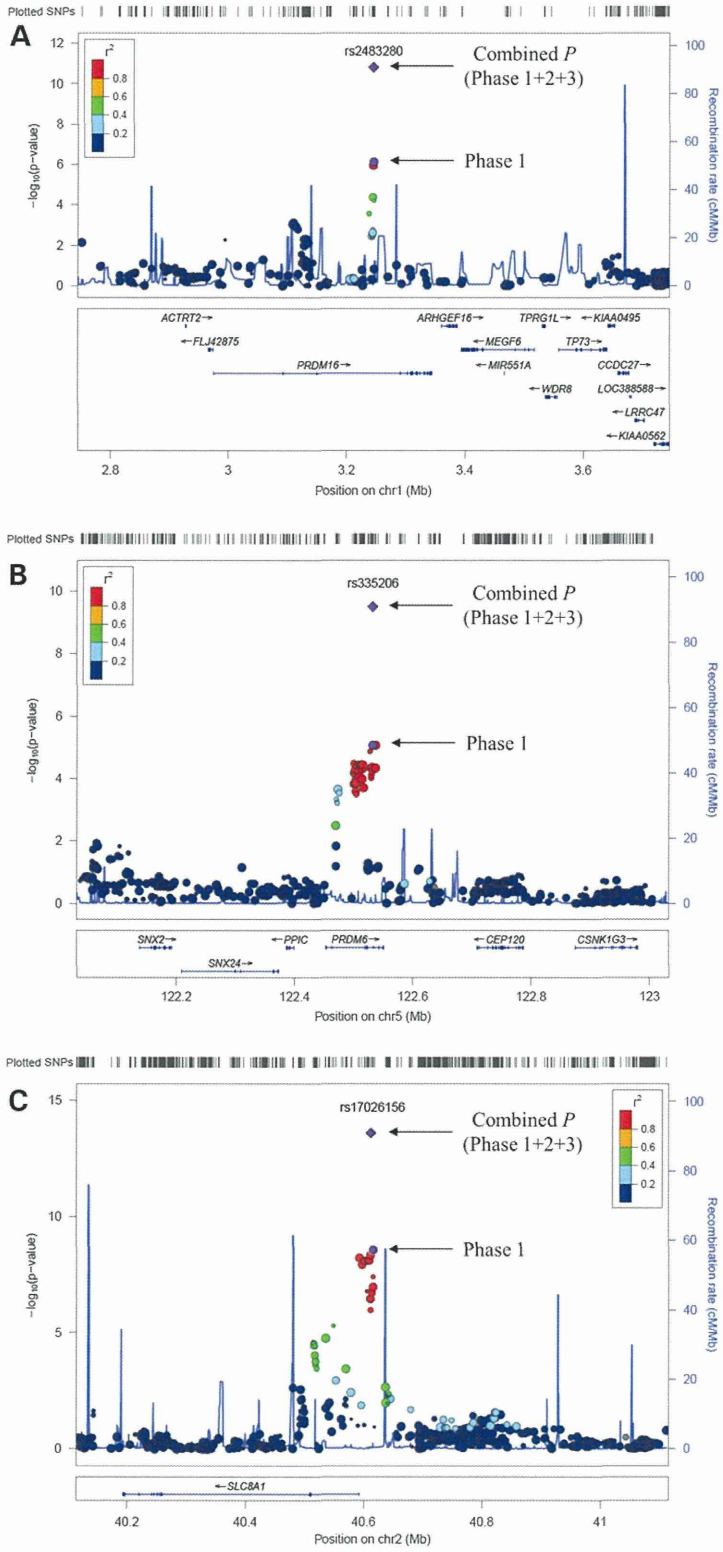

Figure 2. Signal plots for three novel loci across a 1-Mb window. Association of individual SNPs in the Phase 1 study plotted as $-\log10(p)$ against chromosomal base-pair position. The *y*-axis on the right shows the recombination rate, estimated from the HapMap CHB and JPT populations. All *P*-values are from the discovery phase. The purple diamond represents the meta-analysis results of the Phase 1, 2 and 3 studies. (**A**) rs2483280 of PRDM16, (**B**) rs335206 of PRDM6 and (**C**) rs17026156 of SLC8A1.

Table 3. Extension of variants identified in European descendants to Koreans

Reported gene	Ref.	European GWAS SNP	Coded allele	AF	Beta ± SE	*P*-value	Korean GWAS SNP	Coded allele	AF	Beta ± SE	*P*-value
QRS											
NFIA	16	rs9436640	G	0.46	−0.59 ± 0.07	4.57×10^{-18}	rs2103883	C	0.35	−0.57 ± 0.17	6.85×10^{-4}
CRIM1	16	rs7562790	G	0.40	0.39 ± 0.07	8.22×10^{-9}	rs7562790	G	0.65	0.64 ± 0.17	1.52×10^{-4}
HEATR5B-STRN	16	rs17020136	C	0.21	0.51 ± 0.08	1.90×10^{-9}	rs2160411	T	0.55	0.55 ± 0.16	6.76×10^{-4}
HAND1-SAP30L	16	rs13165478	A	0.36	−0.55 ± 0.07	7.36×10^{-14}	rs6580083	G	0.30	−0.85 ± 0.18	1.29×10^{-6}
CDKN1A	14,16	rs9470361	A	0.25	0.87 ± 0.08	3.00×10^{-27}	rs9470366	T	0.15	1.16 ± 0.23	4.02×10^{-7}
VTI1A	16	rs7342028	T	0.27	0.48 ± 0.08	4.95×10^{-10}	rs10885378	G	0.47	0.34 ± 0.16	3.55×10^{-2}
SETBP1	16	rs991014	T	0.42	0.42 ± 0.07	6.2×10^{-10}	rs4890489	A	0.32	0.97 ± 0.17	2.32×10^{-8}
PR											
MEIS1	15	rs11897119	C	0.39	1.36 ± 0.21	4.62×10^{-11}	rs4430933	G	0.76	−1.54 ± 0.47	1.02×10^{-3}
ARHGAP24	14,15	rs7692808	A	0.31	−2.01 ± 0.22	5.99×10^{-20}	rs10012090	A	0.92	−1.87 ± 0.76	1.33×10^{-2}
SOX5	15	rs11047543	A	0.15	−2.09 ± 0.29	3.34×10^{-13}	rs4246224	A	0.14	−1.89 ± 0.58	1.16×10^{-3}
QRS, PR											
EXOG-SCN5A-SCN10A	14–16	rs9851724	C	0.33	−0.66 ± 0.07	1.91×10^{-20}	rs7633988	T	0.29	−0.86 ± 0.18	1.50×10^{-6}
		rs6800541	C	0.40	3.77 ± 0.21	2.10×10^{-74}	rs7433306	C	0.15	4.33 ± 0.56	1.45×10^{-14}
CAV1-CAV2	14,15	rs3807989	A	0.40	3.3	1.10×10^{-4}	rs11773845	C	0.34	0.60 ± 0.17	5.36×10^{-4}
					2.30 ± 0.21	3.66×10^{-28}				3.21 ± 0.42	3.33×10^{-14}
TBX5-TBX3	15,16	rs10850409	A	0.27	−0.49 ± 0.08	3.06×10^{-10}	rs3914956	T	0.49	−0.41 ± 0.16	1.11×10^{-2}
		rs1896312	C	0.28	1.95 ± 0.23	3.13×10^{-17}	rs10744836	T	0.52	1.29 ± 0.40	1.41×10^{-3}

Ref, References; AF, coded allele frequency.

using ENCODE data and the web-based program RegulomeDB. Further, their evolutional conservation was studied by comparing the allelic sequences with primate genome sequences using Ensembl data.

The DNA sequence that encompassed the rs2483280 SNP was predicted to be a ZBTB3 transcription factor-binding motif and an open chromatin region (DNase I-hypersensitive region). However, the SNP sequence was not conserved in primate DNA (Fig. 4A). Thus, we searched for high-LD SNPs near the lead SNP and identified rs2255212 1.5 kb away from the lead SNP (LD score $r^2 = 0.98$ and $D' = 1.00$) (Fig. 4A). rs2255212 was predicted to be a TCF4 transcription factor-binding site and an open chromatin region, and the SNP was highly conserved in all primates. However, the association *P*-value of rs2255212 (1.16×10^{-6}) was not better than that of the lead SNP (rs2483280, $P = 7.47 \times 10^{-7}$). rs335206 was predicted to be a ZNF263-binding site and conserved in all primates, but not an open chromatin region (Fig. 4B).

rs17026156 did not match any functionally conserved sequence, although it was conserved in all primates. Thus, we searched for LD SNPs near the lead SNP and identified a high-LD ($r^2 = 0.93$ and $D' = 1.00$) SNP (rs13017846) that was predicted to be a PIT-1-binding site (Fig. 4C). However, the association *P*-value of rs13017846 (4.47×10^{-9}) was also not better than that of the lead SNP (rs17026156, $P = 2.85 \times 10^{-9}$).

Based on simulation study, the functional variant in a GWAS locus may not have the most significance owing to random sampling. Therefore, functional validation is required to implicate or dismiss rs13017846 and rs2255212, although they did not exceed the significance *P*-values of the lead SNPs.

Notably, the allele frequency of rs17026156 varies widely between ethnicities. The allele frequencies of the ancestral C allele are as low as 0.05 in Europeans (HapMap-CEU) and 0.08 in Africans (HapMap-YRI), whereas they reach as high as 0.57 in Chinese (HapMap-HCB) and 0.35 in Japanese (HapMap-JPT). The rs17026156 was not identified in previous GWASs in European descendants, possibly due to its low allelic frequency in Europeans. Individuals with the C allele of rs17026156 increased PR intervals (beta ± SE, 2.39 ± 0.40 ms) in East Asian population.

Annotation of proximal genes

rs2483280, associated with QRS duration, lies in the third intron of *PRDM16* (PR domain-containing 16), which encodes a protein with a zinc finger DNA-binding domain and PR domain. PRDM16 regulates brown adipose tissue differentiation (20), and its genetic region translocates frequently chromosome 3q21, causing acute myeloid leukemia and myelodysplastic syndrome (21). Recently, fine mapping analysis of 1p36 deletion syndrome implicated a mutation in *PRDM16* as a cause of cardiomyopathy with left ventricular non-compaction and dilated cardiomyopathy, both of which are characterized by progressive cardiac dysfunction, resulting in heart failure, arrhythmia and sudden cardiac death (22).

PRDM16, expressed in the nuclei of cardiomyocytes, potentiates cardiomyocyte proliferation. Haploinsufficiency of *PRDM16* in zebrafish results contractile dysfunction and the reduction of ventricular conduction velocity (22), supporting our finding that rs2483280 in *PRDM16* is associated with QRS duration.

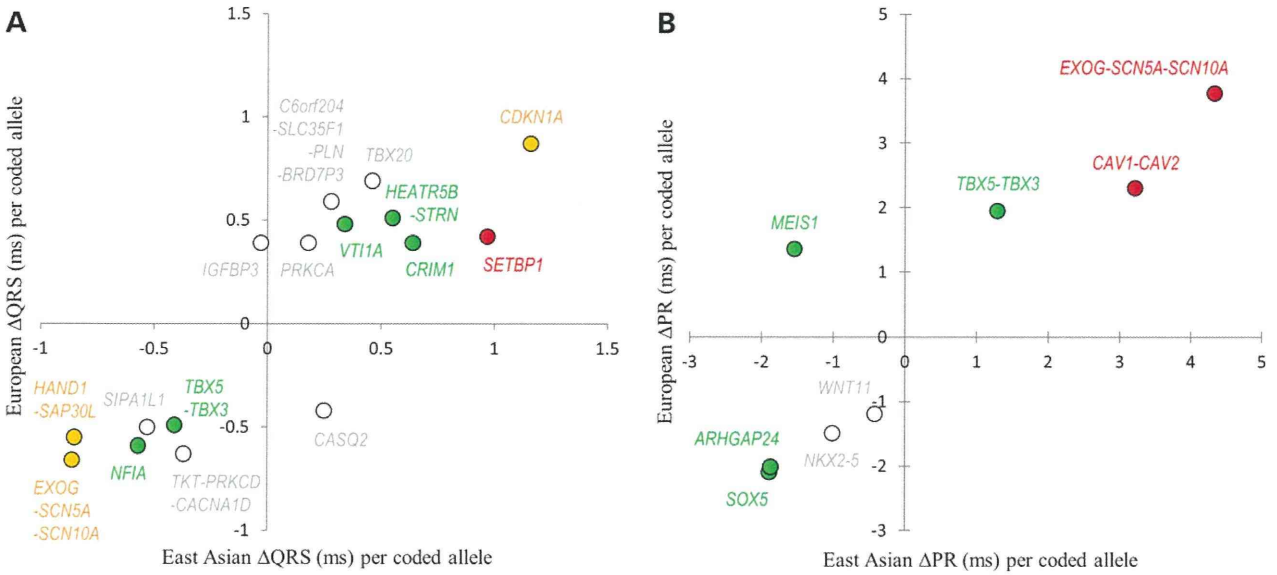

Figure 3. Comparison of effect size (β) of SNPs previously identified in Europeans with those in East Asians. Each dot refers to an association signal, with colors (red, $P < 5 \times 10^{-8}$; orange, $5 \times 10^{-8} \leq P < 10^{-5}$; green, $10^{-5} \leq P < 0.05$; white, $P \geq 0.05$). *CAV1-CAV2* locus was not included in QRS plot because it did not reach genome-wide significance in European GWAS. Effect size was presented beta per copy of the coded allele. (**A**) QRS duration and (**B**) PR interval.

Another novel SNP was associated with QRS duration—rs335206, in the fourth intron of *PRDM6* (PR domain-containing 6), a transcriptional repressor in smooth muscle cells. *PRDM6* regulates the development, differentiation and proliferation of blood vessels (23), and rs335206 was recently linked to systolic blood pressure (24). It is recently reported in mice that *Prdm6* knockout embryos die during development, displaying signs of cardiac insufficiency including a thinning of the myocardial walls (25).

rs17026156, associated with PR interval, lies ~21 kb upstream of *SLC8A1* (sodium/calcium exchanger 1 precursor). SLC8A1 extrudes calcium from cardiac myocytes during relaxation and returns the myocardium to its resting state after excitation (26). Targeted disruption of *SLC8A1* causes defects in heartbeat—*SLC8A1*-/- mouse embryos experience slow and arrhythmic heart contractions (27). We have also reported this locus to correlate with QT interval traits in East Asians (19). Based on the previous reports, *SLC8A1* appears to mediate electrophysiological conductivity during heart.

In conclusion, we have identified three novel loci for QRS duration and PR interval and confirmed 13 previously reported loci. These data will increase our understanding in the genetic architecture that underlies the mechanisms of electrocardiographic traits, QRS duration and PR interval.

MATERIALS AND METHODS

Subjects

The study subjects have been described in a QT interval GWAS (19). Briefly, 6805 subjects from KARE were selected from an ongoing population-based cohort, as part of the Korean Genome and Epidemiology Study (KoGES). Subjects without a self-reported history of cardiac disease, concurrent use of medications that interfere with the ECG measurements and abnormal electrolyte values at the ECG were included. Written informed consent was obtained from all participants, and this project was approved by the institutional review board of the Korea National Institute of Health.

The Phase 2 Japanese subjects were part of the Nagahama Prospective Genome Cohort for Comprehensive Human Bioscience (The Nagahama Study). The Nagahama Study cohort was recruited from 2008 to 2010 from the general population in Nagahama City, a largely rural city of 125 000 inhabitants in Shiga Prefecture that lies in the center of Japan. Of the 9804 participants, persons whose genome-wide SNP was analyzed ($n = 3710$) and who were free of symptomatic cardiovascular disease and abnormal ECG readings ($n = 2285$) were used in the second GWAS panel. All clinical measurements and sampling of blood were performed on enrollment. Genomic DNA was extracted from peripheral blood samples with phenol–chloroform.

The Phase 3 replication panel comprised Japanese from three independent subcohorts. First, the Anti-Aging Center (AAC) cohort included consecutive participants in the medical check-up program at Ehime University Hospital, which was designed specifically to evaluate age-related disorders, including atherosclerosis, cardiovascular disease, physical function and mild cognitive impairment. All clinical data in this study were obtained through the check-up process.

With regard to the second subcohort, the Takashima Study is an ongoing longitudinal study, based on community residents in Takashima City. Takashima City is a semiurban area in Shiga Prefecture, with a population of ~54 000. Study subjects were recruited between 2002 and 2003 from participants of the annual medical check-up program, held by Takashima City. The basic clinical parameters in this study were obtained from the personal medical check-up records of the subjects. The third subcohort of the replication analysis comprised the remaining sample of the Nagahama Study.

All study procedures in Japan were approved by the ethics committee of Ehime University Graduate School of Medicine,