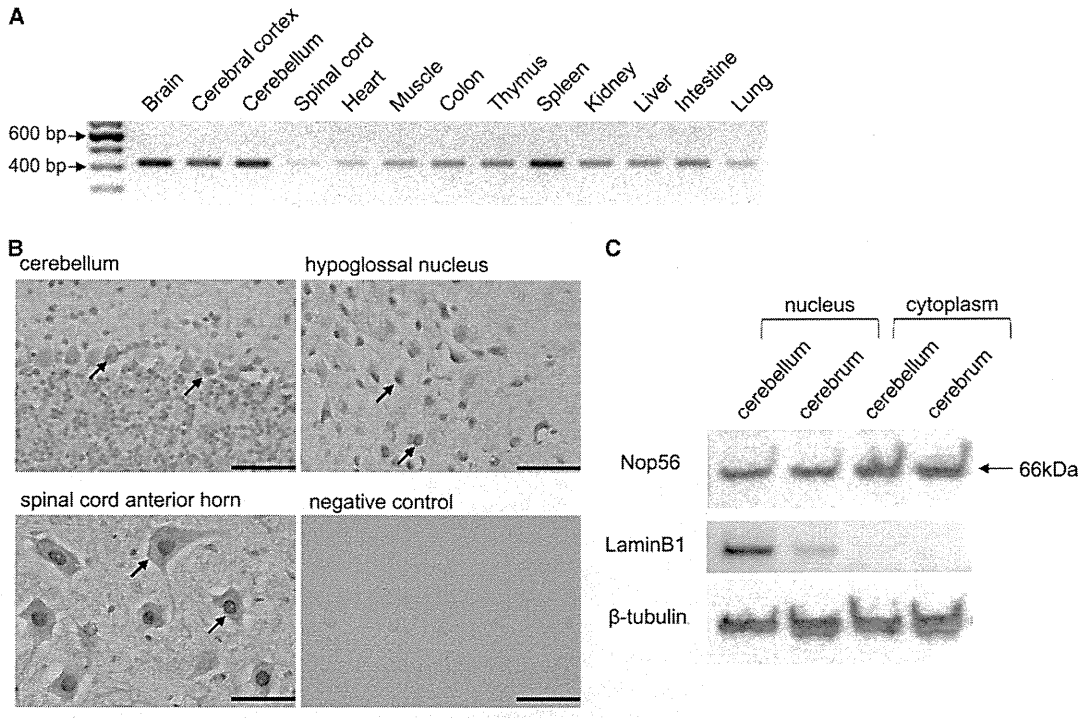

Figure 4. Nop56 in the Mouse Nervous System
(A) RT-PCR analysis of Nop56 (422 bp) in various mouse tissues. cDNA (25 ng) collected from various organs of C57BL/6 mice was purchased from GenoStaf (Tokyo, Japan).
(B) Immunohistochemical analysis of Nop56 in the cerebellum, hypoglossal nucleus, and spinal cord anterior horn in wild-type male Slc:ICR mice at 8 wks of age (Japan SLC, Shizuoka, Japan). The arrows indicate anti- Nop56 antibody staining. The negative control was the cerebellar sample without the Nop56 antibody treatment. Scale bar represents 100 μm.
(C) Immunoblotting of Nop56 (66 kDa) in the cerebellum and cerebrum. Protein sample (10 μg) was subjected to immunoblotting. LaminB1, a nuclear protein, and beta-tubulin were used as loading controls.

We performed fluorescence in situ hybridization to detect RNA foci containing the repeat transcripts in LCLs from patients, as previously described.[17,18] Lymphoblastoid cells from two SCA patients (Ped2_II-2 and Ped5_I-1) and two control subjects were analyzed. An average of 2.1 ± 0.5 RNA foci per cell were detected in 57.0% of LCLs (n = 100) from the SCA subjects through the use of a nuclear probe targeting the GGCCUG repeat, whereas no RNA foci were observed in control LCLs (n = 100) (Figure 6A). In contrast, a probe for the CGCCUG repeat, another repeat sequence in intron 1 of *NOP56*, detected no RNA foci in either SCA or control LCLs (n = 100 each) (Figure 6A), indicating that the GGCCUG repeat was specifically expanded in the SCA subjects. The specificity of the RNA foci was confirmed by sensitivity to RNase A treatment and resistance to DNase treatment (Figure 6A).

Several reports have suggested that RNA foci play a role in the etiology of SCA through sequestration of specific RNA-binding proteins.[5–7] In silico searches (ESEfinder 3.0) predicted an RNA-binding protein, SRSF2 (MIM 600813), as a strong candidate for binding of the GGCCUG repeat. Double staining with the probe for the GGCCUG repeat and an anti-SRSF2 antibody (Sigma-Aldrich, Tokyo, Japan) was performed. The results showed colocalization of RNA foci with SRSF2, whereas NOP56 and coilin were not colocalized with the RNA foci (Figure 6B), suggesting a specific interaction of endogenous SRSF2 with the RNA foci in vivo.

To further confirm the interaction, gel-shift assays were carried out for investigation of the binding activity of SRSF2 with $(GGCCUG)_n$. Synthetic RNA oligonucleotides (200 pmol), $(GGCCUG)_4$ or $(CUG)_6$, which is the latter part of the hexanucleotide, as well as the repeat RNA involved in myotonic dystrophy type 1 (DM1 [MIM 160900])[18] and SCA8 (MIM 608768),[5] were denatured and immediately mixed with different amounts (0, 0.2, or 0.6 μg) of recombinant full-length human SRSF2 (Abcam, Cambridge, UK). The mixtures were incubated, and the protein-bound probes were separated from the free forms by electrophoresis on 5%–20% native polyacrylamide gels. The separated RNA probes were detected with SYBR Gold staining (Invitrogen, Carlsbad, CA, USA). We found a strong association of $(GGCCUG)_4$ with SRSF2 in vitro in comparison to $(CUG)_6$ (Figure 6C). Collectively, we concluded that (GGCCUG)n interacts with SRSF2.

It is notable that *MIR1292* is located just 19 bp 3′ of the GGCCTG repeat (Figure 2D). MiRNAs such as *MIR1292* are small noncoding RNAs that regulate gene expression by inhibiting translation of specific target mRNAs.[19,20] MiRNAs are believed to play important roles in key molecular

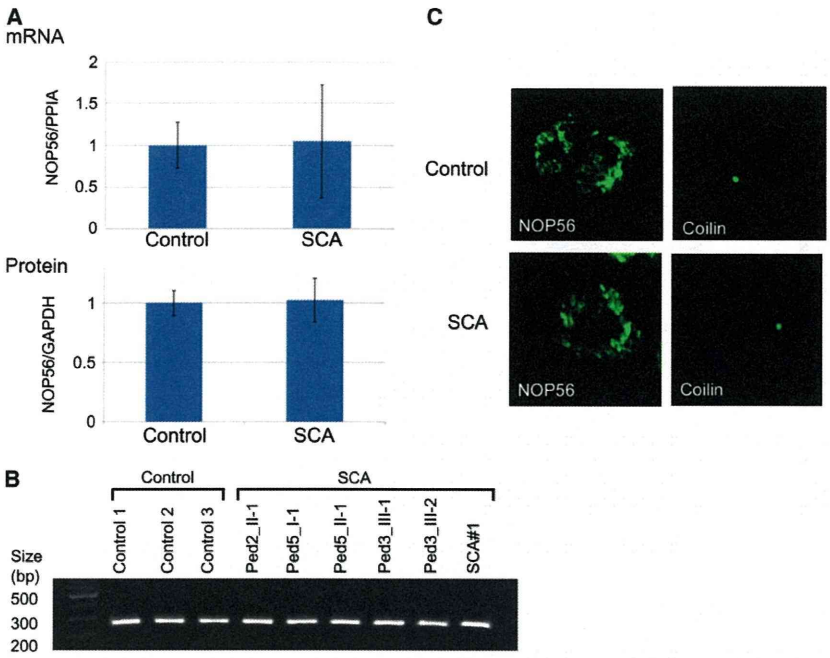

Figure 5. Analysis of NOP56 in LCLs from SCA Patients

(A) mRNA expression (upper panel) and protein levels (lower panel) in LCLs from cases (n = 6) and controls (n = 3) were measured by RT-PCR and immunoblotting, respectively. cDNA (10 ng) was transcribed from total RNA isolated from LCLs and used for RT-PCR. Immunoblotting was performed with the use of a protein sample (40 μg) extracted from LCLs. The data indicate the mean ± SD relative to the levels of *PP1A* and GAPDH, respectively. There was no significant difference between LCLs from controls and cases.

(B) Analysis for splicing variants of NOP56 cDNA. RT-PCR with 10 ng of cDNA and primers corresponding to the region from the 5′ UTR to exon 4 around the repeat expansion was performed. The PCR product has an expected size of 230 bp.

(C) Immunocytochemistry for NOP56 and coilin. Green signals represent NOP56 or coilin. Shown are representative samples from 100 observations of controls or cases.

pathways by fine-tuning gene expression.[19,20] Recent studies have revealed that miRNAs influence neuronal survival and are also associated with neurodegenerative diseases.[21,22] In silico searches (Target Scan Human 5.1) predicted glutamate receptors (*GRIN2B* [MIM 138252] and *GRIK3* [MIM 138243]) to be potential target genes. Real-time RT-PCR using TaqMan probes for miRNA (Invitrogen, Carlsbad, CA, USA) revealed that the levels of both mature and precursor *MIR1292* were significantly decreased in SCA LCLs (Figure 6D), indicating that the GGCCTG repeat expansion decreased the transcription of *MIR1292*. A decrease in *MIR1292* expression may upregulate glutamate receptors in particular cell types; e.g., *GRIK3* in stellate cells in the cerebellum,[23] leading to ataxia because of perturbation of signal transduction to the Purkinje cells. In addition, it has been suggested, on the basis of ALS mouse models,[24,25] that excitotoxicity mediated by a type of glutamate receptor, the NMDA receptor including *GRIN2B*, is involved in loss of spinal neurons. A very slowly progressing and mild form of the motor neuron disease, such as that described here, which is limited to mostly fasciculation of the tongue, limbs and trunk, may also be compatible with such a functional dysregulation rather than degeneration.

In the present study, we have conducted genetic analysis to find a genetic cause for the unique SCA with motor neuron disease. With extensive sequencing of the 1.8 Mb linked region, we found large hexanucleotide repeat expansions in *NOP56*, which were completely segregated with SCA in five pedigrees and were found in four unrelated cases with a similar phenotype. The expansion was not found in 300 controls or in other neurodegenerative diseases. We further proved that repeat expansions of *NOP56* induce RNA foci and sequester SRSF2. We thus concluded that hexanucleotide repeat expansions are considered to cause SCA by a toxic RNA gain-of-function mechanism, and we name this unique SCA as SCA36. Haplotype analysis indicates that hexanucleotide expansions are derived from a common ancestor. The prevalence of SCA36 was estimated at 3.6% in the SCA cohort in Chugoku district, suggesting that prevalence of SCA36 may be geographically limited to the western part of Japan and is rare even in Japanese SCAs.

Expansion of tandem nucleotide repeats in different regions of respective genes (most often the triplets CAG and CTG) has been shown to cause a number of inherited diseases over the past decades. An expansion in the coding region of a gene causes a gain of toxic function and/or reduces the normal function of the corresponding protein at the protein level. RNA-mediated noncoding repeat expansions have also been identified as causing eight other neuromuscular disorders: DM1, DM2 (MIM 602668), fragile X tremor/ataxia syndrome (FXTAS [MIM 300623]), Huntington disease-like 2 (HDL2 [MIM 606438]), SCA8, SCA10 (MIM 603516), SCA12 (MIM 604326), and SCA31 (MIM 117210).[26] The repeat numbers in affected alleles of SCA36 are among the largest seen in this group of diseases (i.e., there are thousands of repeats). Moreover, SCA36 is not merely a nontriplet repeat expansion disorder similar to SCA10, DM2, and SCA31, but is now proven to be a human disease caused by a large hexanucleotide repeat expansion. In addition, no or only weak anticipation has been reported for noncoding repeat expansion in SCA, whereas clear anticipation has been reported for most polyglutamine expansions in SCA.[2] As such, absence of anticipation in SCA36 is in accord with previous studies

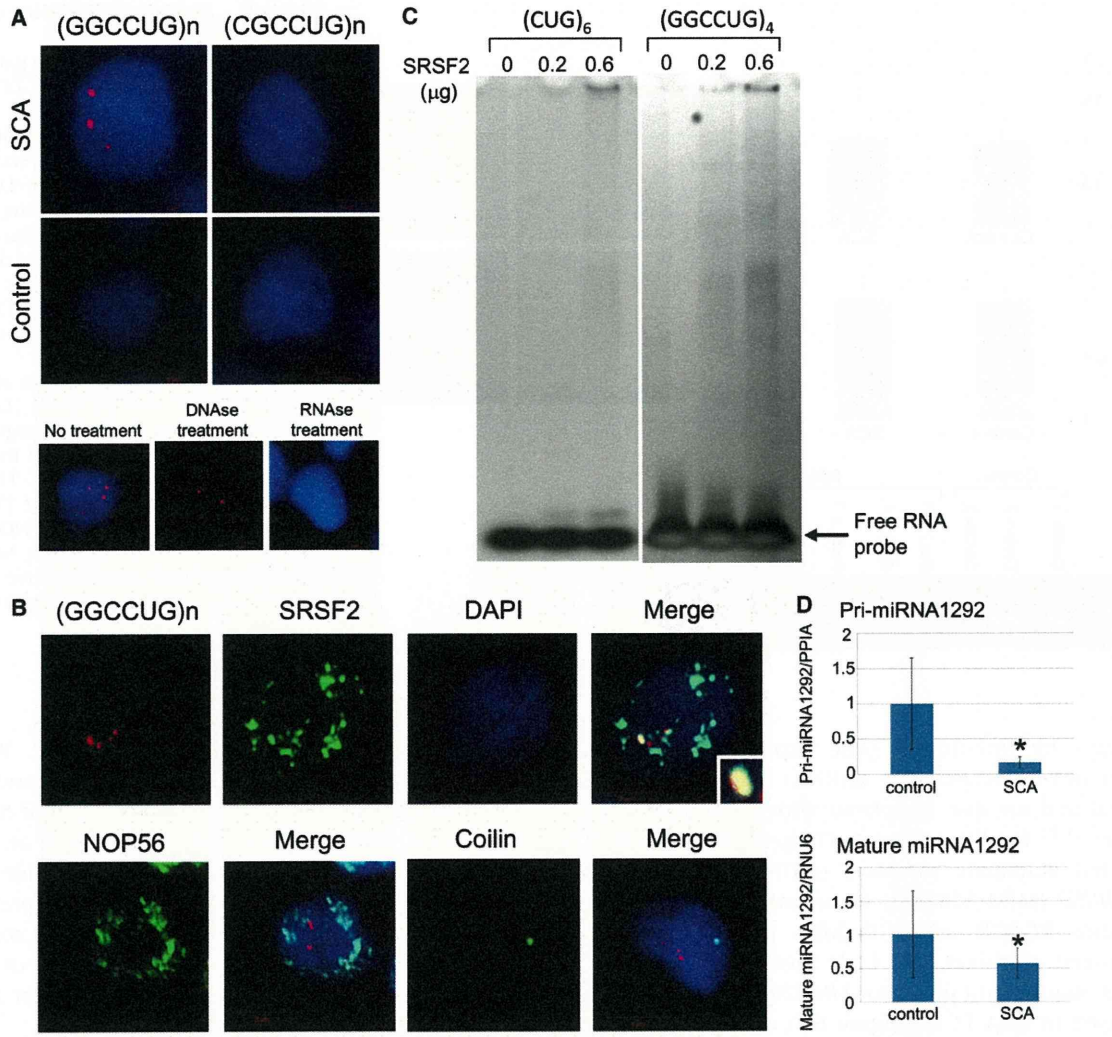

Figure 6. RNA Foci Formation and Decreased Transcription of *MIR1292*

(A) Cells were fixed on coverslips and then hybridized with solutions containing either a Cy3-labeled $C(CAGGCC)_2CAG$ or $G(CAGGCG)_2CAG$ oligonucleotide probe (1 ng/μl). For controls, the cells were treated with 1000 U/ml DNase or 100 μg/ml RNase for 1 hr at 37°C prior to hybridization, as indicated. After a wash step, coverslips were placed on the slides in the presence of ProLong Gold with DAPI mounting media (Molecular Probes, Tokyo, Japan) and photographed with a fluorescence microscope. The upper panels indicate LCLs from an SCA case and a control hybridized with $C(CAGGCC)_2CAG$ (left) or $G(CAGGCG)_2CAG$ (right). Red and blue signals represent RNA foci and the nucleus (DAPI staining), respectively. Similar RNA foci formation was confirmed in LCLs from another index case. The lower panels show RNA foci in SCA LCLs treated with DNase or RNase.

(B) Double staining was performed with the probe for $(GGCCUG)_n$ (red) and anti-SRSF2, NOP56, or coilin antibody (green).

(C) Gel-shift assays revealed specific binding of SRSF2 to $(GGCCUG)_4$ but little to $(CUG)_6$.

(D) RNA samples (10 ng) were extracted from LCLs of controls ($n = 3$) and cases ($n = 6$). MiRNAs were measured with the use of a TaqMan probe for precursor (Pri-) and mature *MIR1292*. The data indicate the mean ± SD, relative to the levels of *PP1A* or *RNU6*. *: $p < 0.05$.

on SCAs with noncoding repeat expansions. The common hallmark in these noncoding repeat expansion disorders is transcribed repeat nuclear accumulations with respective repeat RNA-binding proteins, which are considered to primarily trigger and develop the disease at the RNA level. However, multiple different mechanisms are likely to be involved in each disorder. There are at least two possible explanations for the motor neuron involvement of SCA36: gene- and tissue-specific splicing specificity of *SRSF2* and involvement of miRNA. In SCA36, there is the possibility that the adverse effect of the expansion mutation is mediated by downregulation of miRNA expression. The biochemical implication of miRNA involvement cannot be evaluated in this study, because availability of tissue samples from affected cases was limited to LCLs. Given definitive downregulation of miRNA 1292 in LCLs, we should await further study to substantiate its involvement in affected tissues. Elucidating which mechanism(s) plays a critical role in the pathogenesis will be required for determining whether cerebellar degeneration and motor neuron disease occur through a similar scenario.

In conclusion, expansion of the intronic GGCCTG hexanucleotide repeat in *NOP56* causes a unique form of SCA, SCA36, which shows not only ataxia but also motor neuron dysfunction. This characteristic disease phenotype can be explained by the combination of RNA gain of function and *MIR1292* suppression. Additional studies are required to investigate the roles of each mechanistic component in the pathogenesis of SCA36.

Supplemental Data

Supplemental Data include one figure and three tables and can be found with this article online at http://www.cell.com/AJHG/.

Acknowledgments

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Web Resources

The URLs for data presented herein are as follows:

ESEfinder 3.0, http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home
NCBI, http://www.ncbi.nlm.nih.gov/
Target Scan Human 5.1, http://www.targetscan.org/
UCSC Genome Bioinformatics, http://genome.ucsc.edu

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Neurourology and Urodynamics

B-Type Natriuretic Peptide as an Independent Correlate of Nocturnal Voiding in Japanese Women

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Aims: To investigate whether objective cardiovascular parameters have an independent association with nocturnal voiding in women. **Methods:** Thirty-two parameters derived from questionnaires, and anthropometric, physiological and biochemical measures of 5,980 women were applied for analysis. Nocturnal voiding was assessed by the International Prostate Symptom Score and the Overactive Bladder Symptom Score. We measured variables including previously reported correlates of nocturnal voiding, such as age, a history of hypertension, and a history of diabetes, as well as those focusing on cardiovascular function, such as the cardio-ankle vascular index, the augmentation index, the ankle-brachial index, plasma B-type natriuretic peptide (BNP), and C-reactive protein (CRP). **Results:** Age [odds ratio (OR): 1.058, $P < 0.001$], length of sleep (OR: 1.194, $P < 0.001$), sleeplessness (OR: 2.841, $P < 0.001$), urgency (OR: 1.528, $P < 0.001$), log(BNP) (OR: 2.031, $P < 0.001$), waist circumference (OR: 1.037, $P = 0.002$), body mass index (OR: 0.935, $P = 0.007$), menopause (OR: 1.503, $P = 0.043$), and history of hypertension (OR: 1.225, $P = 0.029$) were independently associated with nocturnal voiding ≥ 2 times. Age ($\beta = 0.256$, $P < 0.001$), urgency ($\beta = 0.195$, $P < 0.001$), sleeplessness ($\beta = 0.181$, $P < 0.001$), length of sleep ($\beta = 0.088$, $P < 0.001$), log(BNP) ($\beta = 0.072$, $P < 0.001$), waist circumference ($\beta = 0.086$, $P < 0.001$), and low-density lipoprotein-cholesterol ($\beta = -0.038$, $P = 0.003$) were significantly correlated with the severity of nocturnal voiding. **Conclusions:** Plasma BNP, which represents cardiac load, is strongly associated with the prevalence and severity of nocturnal voiding in Japanese women, as well as previously known correlates including age, urgency, quality and quantity of sleep, and obesity. *Neurourol. Urodynam.*

Key words: B-type natriuretic peptide; epidemiology; nocturia; nocturnal voiding

INTRODUCTION

Nocturia is one of the most bothersome lower urinary tract symptoms (LUTS) in the elderly.[1] The causes of this symptom are multifold and are usually attributable to decreased nocturnal bladder capacity, increased nocturnal urine volume, and/or sleep problems.[2] Previous epidemiological studies have demonstrated that there are many correlates to nocturnal voiding, which include a past history of hypertension, diabetes, stroke, arrhythmia, sleeplessness, urinary urgency, and a large body mass index (BMI).[2,3] Several of these factors are components of metabolic syndrome (METS), and are related to cardiovascular disease (CVD).

Although each component of METS, hypertension, diabetes, dyslipidemia, and obesity, is an independent risk factor of CVD, synergistic effects as a risk of CVD are observed if combined.[4] Several studies have described the components of METS plus smoking as "vascular risk factors," and have reported the association between LUTS and these factors.[5,6] Therefore, it has been suggested that nocturnal voiding has a relationship with METS and/or cardiovascular conditions. However, no previous studies have shown a direct association between LUTS including nocturnal voiding and objective cardiovascular conditions, which could be assessed using several parameters including blood pressure (diastolic, systolic, and central), heart rate, the cardio-ankle vascular index (CAVI), augmentation index (AI), and ankle-brachial index (ABI).

The main purpose of this study was to investigate whether various objective parameters representing cardiovascular conditions are independent correlates for the prevalence and severity of nocturnal voiding when analyzed with previously known correlates, using data from a community-indwelling population in Japan.

Eric Rovner led the review process.
Conflict of interest: none.
†See Appendix.
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SUBJECTS AND METHODS

The Nagahama cohort project is a population-based prospective cohort survey of a broad range of chronic illness, which was conducted in Nagahama City, Shiga prefecture, Japan.[7] This is a prospective study composed of a questionnaire survey, anthropometric measures, physiological measures, biochemical measures from blood samples, and genomic information. The baseline data of the study population were obtained from August 2008 to November 2010. All protocols and informed consent procedures were approved by the Kyoto University Graduate School and Faculty of Medicine Ethics Committee, the Ad hoc Review Board of the Nagahama Cohort Project, and the Nagahama Municipal Review Board of Personal Information Protection.

The International Prostate Symptom Score (I-PSS)[8,9] and the Overactive Bladder Symptom Score[10] were used to assess LUTS. The Japanese versions of these questionnaires have been validated.[9,10] Frequency of nocturnal voiding was assessed using the seventh question of the I-PSS and the second question of the OABSS. For analyses, we only used data from those participants whose answers for these two questions were compatible with each other. Self-reported information on medical history, major comorbidities, current medication use, lifestyle and psychosocial factors were also collected. Researchers asked a participant to answer appropriately to minimize the lack of data, if an answer sheet was incomplete with some blanks.

For the analysis of the prevalence of nocturia, we defined nocturic subjects as those who voided urine twice or more during the night. If some other data, including fasting blood sugar levels, were lacking, we excluded these data for analyses. We also stratified the subjects into those without nocturnal voiding, those with one nocturnal voiding, those with two nocturnal voidings, and those with three or more nocturnal voidings in the analysis for severity of nocturnal voiding. Independent variables investigated by questionnaire in this study were menopause, smoking, urgency (the third question of the OABSS), time in bed, sleeplessness, daytime sleepiness, history of hypertension, history of diabetes, history of dyslipidemia, history of cerebrovascular disease, and history of CVD.

Height, weight, and waist circumference were measured, and BMI was calculated. Physiological variables applied in this study were blood pressure (systolic, diastolic, and central), heart rate, AI, CAVI, and ABI. These variables were obtained twice 5-min apart, and were averaged. Central blood pressure and AI were measured using the HEM-9000AI® (Omron Corp., Kyoto, Japan), and CAVI and ABI were measured using the VaSera® VS-1000 (Fukuda Denshi Inc., Tokyo, Japan). The value of AI was modified under the setting of heart rate as 75 beats/min. Central blood pressure and AI represent the stiffness of the systemic arterial tree and the cardiac load, and they were reported to predict prognosis in subjects with end-stage renal disease or coronary heart disease.[11] CAVI is a marker of central arterial stiffness, and several studies showed this parameter can predict the prognosis of subjects with acute coronary syndrome, end-stage renal disease, or heart failure.[11,12] ABI is an index of stenosis of the central artery, and can predict CVDs.[13]

Blood samples were obtained in the fasting period. Data of participants whose blood samples were obtained within 8 hr after final ingestion were excluded from analysis because of inappropriate fasting blood sugar. Biochemical variables applied in this study other than fasting blood sugar were hemoglobin A1c, insulin, triglycerides, low density lipoprotein-cholesterol (LDLC), high-density lipoprotein-cholesterol (HDLC), B-type natriuretic peptide (BNP), hypersensitive C-reactive protein (CRP), creatinine, and cholinesterase. Since the distributions of BNP and CRP were skewed, natural log transformations of these two variables were used for analyses. The estimated glomerular filtration rate (eGFR) was calculated according to the Modification of Diet in Renal Diseases Study equations.[14,15]

Statistics

The total number of independent variables was 32, and of those, 22 were continuous variables and 9 were nominal variables. Only the parameter of daytime sleepiness was ordinal, ranked 1–5 (Table I). Dependent variables were a nocturnal voiding frequency of two or more times as defined for the analysis of the prevalence of nocturnal voiding, and the stratification of nocturnal voiding frequency in the analysis of its severity.

For analyzing correlates of the prevalence of nocturnal voiding, statistical analyses were performed in three steps using a logistic regression model. First, bivariate analysis was performed to confirm the basic relationship between each independent variable and nocturnal voiding, and if $P < 0.25$, the variable was applied to the next step. In step 2, multivariate logistic regression analysis was performed using the parameters included in the same category; for example, diabetic history, blood sugar, hemoglobin A1c, and serum insulin levels were used in the category of diabetes, to remove confounding factors. The types of categories with multiple variables were sleep, obesity, hypertension, diabetes, dyslipidemia, cardiac function, arteriosclerosis, and renal function. Using the variables with $P < 0.05$ in step 2, final multivariate logistic regression analysis was performed. If a category included only one variable, such as cerebral vascular disease, step 2 analysis was skipped, and $P < 0.25$ in step 1 would be required for final analyses. For analyzing correlates of the severity of nocturnal voiding, we used multiple regression analysis. All P-values were two-sided and $P < 0.05$ was considered significant. SPSS® version 13.0 was used for all calculations.

RESULTS

A total of 6,581 female residents participated in the Nagahama project. Of these, 482, 131, and 18 residents were excluded from this study because of inconsistency of answers for two questions regarding nocturnal voiding, a non-fasting blood sugar examination, and insufficient data, respectively. The data of the remaining 5,980 (91%) residents were used in this study.

Characteristics of Participants

The characteristics and the distributions of the participants are shown in Table I. The prevalence of none, one, two, and three or more nocturnal voiding episodes was 49.0%, 38.5%, 9.9%, and 2.6%, respectively.

Correlates for the Prevalence of Nocturnal Voiding

In step 1 of the analysis, only one variable, serum insulin level, was eliminated from step 2. In step 2, the four variables of history of hypertension, history of diabetes, HDLC, and serum creatinine level, were eliminated from step 3. Finally, age, urgency, time in bed, sleeplessness, waist circumference, log(BNP), BMI, menopause, and history of hypertension were significantly and independently associated with nocturnal

TABLE I. Background of Participants and Types of Variables

Category	Factor	Type of variable	Number (rate)	Mean (SD)
Subjects without nocturnal voiding			2,932 (49.0%)	
Subjects with nocturnal voiding			3,048 (51.0%)	
One nocturnal voiding			2,303 (38.5%)	
Two nocturnal voidings			591 (9.9%)	
Three or more nocturnal voidings			154 (2.6%)	
Age	Age	Continuous		52.6 (13.2)
Hormonal status	Menopause (yes)	Nominal	3,573 (59.7%)	
Smoking	Smoking (yes)	Nominal	385 (6.4%)	
Urgency	Urgency (yes)	Nominal	2,727 (54.6%)	
Sleep	Time in bed (hr)	Continuous		6.48 (1.03)
	Sleeplessness (yes)	Nominal	1,051 (17.6%)	
	Daytime sleepiness (yes)	Ordinal		
Obesity	Body mass index (kg/m/m)	Continuous		21.7 (3.2)
	Waist circumference (cm)	Continuous		78.6 (9.2)
Hypertension	History (yes)	Nominal	972 (16.3%)	
	Systolic pressure (mmHg)[a]	Continuous		120.4 (17.7)
	Diastolic pressure (mmHg)[a]	Continuous		73.8 (10.9)
	Central systolic pressure (mmHg)[a]	Continuous		125.8 (20.0)
Diabetes	History (yes)	Nominal	182 (3.0%)	
	Blood sugar (mg/dl)	Continuous		88.7 (12.4)
	Hemoglobin A1c (%)	Continuous		5.08 (0.45)
	Insulin (μU/ml)	Continuous		5.24 (9.03)
Dyslipidemia	History (yes)	Nominal	663 (11.1%)	
	Triglycerides (mg/dl)	Continuous		86.5 (52.5)
	Low-density lipoprotein-cholesterol (mg/dl)	Continuous		122.8 (31.2)
	High-density lipoprotein-cholesterol (mg/dl)	Continuous		68.8 (16.3)
Cerebrovascular disease	History (yes)	Nominal	39 (0.7%)	
Cardiac functions	History of cardiovascular disease (yes)	Nominal	270 (4.5%)	
	Heart rate (/min)[a]	Continuous		64.4 (10.0)
	B-type natriuretic peptide (pg/ml)	Continuous		17.5 (18.7)
Arterioscrelosis	C-reactive protein (ng/ml)	Continuous		762.5 (290.3)
	Augmentation index (%)[a]	Continuous		80.8 (11.5)
	Cardio-ankle vascular index[a]	Continuous		7.20 (1.04)
	Ankle-brachial index[a]	Continuous		1.08 (0.08)
Renal functions	Serum creatinine (mg/dl)	Continuous		0.62 (0.18)
	Estimated glomerular filtration rate (ml/min)	Continuous		77.6 (15.3)
Hepatic function	Serum cholinesterase (IU/L)	Continuous		328.8 (78.4)

[a]The average value of two measurements per individual.

voiding ≥2 times. BMI had an inverse association with the prevalence of nocturnal voiding after the final step. While bivariate analysis showed a positive correlation of BMI with the prevalence of nocturnal voiding [odds ratio (OR): 1.073, $P < 0.001$], waist circumference-adjustment changed the correlation from positive to negative. Central blood pressure, AI, CAVI, ABI, and CRP had no independent association (Table II).

Correlates for the Severity of Nocturnal Voiding

In this analysis, the adjusted r square value, the standard error, and the *F*-value were 0.271, 0.674, and 71.1, respectively. Age, urgency, sleeplessness, length of sleep, waist circumference, log(BNP), history of hypertension, and LDLC were significantly correlated with the severity of nocturnal voiding. LDLC had an inverse association with the severity of nocturnal voiding. While bivariate analysis showed a positive correlation of LDLC with the severity of nocturnal voiding ($\beta = 0.097$, $P < 0.001$), age-adjustment changed the correlation from positive to negative. Similar to the prevalence of nocturnal voiding, central blood pressure, AI, CAVI, ABI, and CRP had no independent association (Table III).

DISCUSSION

Many epidemiological studies have revealed a close association between nocturnal voiding and hypertension, diabetes, and obesity, which are components of METS.[3] Several other studies have reported associations between LUTS and vascular risk factors.[5,6] Therefore, it has been suggested that nocturnal voiding might have a relationship with cardiovascular conditions. Currently, several types of cardiovascular conditions are able to be assessed with various parameters. Cardiac load is represented by BNP and AI.[11,16] AI and central blood pressure are closely associated with each other, and it has been reported that central blood pressure is an independent risk factor of cardiovascular events.[11] Arterial stiffness, which results from fibrosis of the media of elastic arteries, can be estimated by pulse wave velocity, and CAVI is one of the indices to assess pulse wave velocity.[12] AI also reflects arterial stiffness. ABI is an index for arteriostenosis, which occurs at elastic and muscular arteries.[13] Hypersensitive-CRP is considered to mainly originate from endothelial and adipose cells, and to represent systemic inflammation inducing atherosclerosis.[17]

TABLE II. Correlates for the Prevalence of Nocturnal Voiding (≥2 Time)

		Step 1	Step 2	Step 3	
Category	Factor	*P*-value	*P*-value	*P*-value	OR (95% CI)
Age	Age	<0.001	—	<0.001	1.058 (1.044–1.079)
Hormonal status	Menopause (yes/no)	<0.001	—	0.043	1.503 (1.013–2.232)
Smoking	Smoking (yes/no)	<0.001	—	0.33	
Urgency	Urgency (yes/no)	<0.001	—	<0.001	1.528 (1.416–1.648)
Sleep	Time in bed (hr)	0.143	0.001	<0.001	1.194 (1.096–1.300)
	Sleeplessness (yes/no)	<0.001	0.002	<0.001	2.841 (2.347–3.436)
	Daytime sleepiness (yes/no)	0.009	<0.001	0.59	
Obesity	Body mass index (kg/m/m)	<0.001	<0.001	0.007	0.935 (0.890–0.982)
	Waist circumference (cm)	<0.001	<0.001	0.002	1.037 (1.019–1.055)
Hypertension	History (yes/no)	<0.001	<0.001	0.049	1.225 (1.001–1.500)
	Systolic pressure (mmHg)	<0.001	0.66	Con	
	Diastolic pressure (mmHg)	<0.001	<0.001	0.46	
	Central systolic pressure (mmHg)	<0.001	<0.001	0.85	
Diabetes	History (yes/no)	<0.001	0.36	Con	
	Blood sugar (mg/dl)	<0.001	<0.001	0.15	
	Hemoglobin A1c (%)	<0.001	<0.001	0.88	
	Insulin (μU/ml)	0.49	NA	NA	
Dyslipidemia	History (yes/no)	<0.001	<0.001	0.70	
	Triglycerides (mg/dl)	<0.001	<0.001	0.17	
	Low-density lipoprotein-cholesterol (mg/dl)	<0.001	<0.001	0.09	
	High-density lipoprotein-cholesterol (mg/dl)	0.004	0.32	Con	
Cerebrovascular disease	History (yes/no)	0.070		0.42	
Cardiac functions	History of cardiovascular disease (yes/no)	<0.001	<0.001	0.23	
	Heart rate (/min)	0.062	<0.001	0.17	
	B-type natriuretic peptide (pg/ml)[a]	<0.001	<0.001	<0.001	2.031 (1.529–2.698)
Arterioscrelosis	C-reactive protein (ng/ml)[a]	<0.001	<0.001	0.22	
	Augmentation index (%)	<0.001	0.004	0.38	
	Cardio-ankle vascular index	<0.001	<0.001	0.15	
	Ankle-brachial index	<0.001	<0.001	0.56	
Renal functions	Serum creatinine (mg/dl)	0.078	<0.001	0.88	
	Estimated glomerular filtration rate (ml/min)	<0.001	<0.001	0.06	
Hepatic function	Serum cholinesterase (IU/L)	<0.001	—	0.65	

NA, not analyzed; Con, not included in the final analysis because of severe confounding in the step 2 analysis.
[a]Natural log transformations of the BNP and CRP levels were used for analysis.

In this study, several variables, such as age, urgency, history of hypertension, obesity, and sleep-related factors, remained independent, as reported previously. As well as these known correlates, our study revealed that BNP was another strong independent correlate of nocturnal voiding. Plasma BNP levels have previously been reported to be closely related to the nocturnal polyuria index,[18] and this observation is compatible with that of another study, which found that nocturnal polyuria results from natriuresis during the night.[19] Since BNP represent cardiac load, as mentioned above, this suggests that clinical and preclinical heart failure might be an important cause of nocturnal voiding, and could also be a potential target of treatment for nocturnal voiding.

Unlike the significant correlation of BNP with nocturnal voiding in our study, CAVI or ABI did not have an independent association with the prevalence or severity of nocturnal voiding, although they exhibited a significant relationship until step 2 in the analysis of correlates for the prevalence of nocturnal voiding. This observation suggests that current status of arterial stiffness and arteriostenosis of elastic and muscular arteries have little, if any, impact on the frequency of nocturnal voiding.

Among the biochemical examinations investigated in this study, CRP and LDLC are associated with atherosclerosis.[17] While more than 30 epidemiological studies have reported that higher levels of CRP are a significant independent risk factor of ischemic stroke and cardiovascular events, some recent studies have reported that elevated serum CRP levels also have an association with several types of LUTS.[20–22] Although log(CRP) remained significantly associated with the prevalence of nocturnal voiding until step 2 of the analysis, it lost statistical significance in the final step of our study. Meanwhile, LDLC had an inverse association with the severity of nocturnal voiding. Since bivariate analysis demonstrated a positive correlation between LDLC and the severity of nocturnal voiding, this observation is probably due to the strong correlation between LDLC and aging.

Similarly, BMI was independently and inversely associated with prevalence of nocturnal voiding ≥2 times. There are many previous epidemiological studies that have shown a relationship between a higher BMI and nocturnal voiding. Most of these studies examined only BMI, but not waist circumference. BMI had a positive association with the prevalence of nocturnal voiding on bivariate analysis, and the outcome in the final step resulted from a strong correlation between BMI and waist circumference. Based on our observations of the prevalence and severity of nocturnal voiding, it is suggested that central obesity assessed by waist circumference plays a more important role in the development of nocturnal voiding than total obesity assessed by BMI of Japanese women.

TABLE III. Correlates for the Severity of Nocturnal Voiding

Factor	Beta-coefficient	*P*-value
(Constant)		<0.001
Age	0.256	<0.001
Menopause (yes/no)	—	0.12
Smoking (yes/no)	—	0.59
Urgency (yes/no)	0.195	<0.001
Time in bed (hr)	0.088	<0.001
Sleeplessness (yes/no)	0.181	<0.001
Daytime sleepiness (yes/no)	—	0.79
Body mass index (kg/m/m)	—	0.06
Waist circumference (cm)	0.086	<0.001
History of hypertension (yes/no)	0.051	<0.001
Systolic pressure (mmHg)	—	0.11
Diastolic pressure (mmHg)	—	0.06
Central systolic pressure (mmHg)	—	0.61
History of diabetes (yes/no)	—	0.57
Blood sugar (mg/dl)	—	0.20
Hemoglobin A1c (%)	—	0.75
Insulin (μU/ml)	—	0.09
History of dyslipidemia (yes/no)	—	0.29
Triglycerides (mg/dl)	—	0.56
Low-density lipoprotein-cholesterol (mg/dl)	−0.038	0.003
High-density lipoprotein-cholesterol (mg/dl)	—	0.67
History of cerebrovascular disease (yes/no)	—	0.32
History of cardiovascular disease (yes/no)	—	0.25
Heart rate (/min)	—	0.12
B-type natriuretic peptide (pg/ml)[a]	0.072	<0.001
C-reactive protein (ng/ml)[a]	—	0.13
Augmentation index (%)	—	0.10
Cardio-ankle vascular index	—	0.58
Ankle-brachial index	—	0.67
Serum creatinine (mg/dl)	—	0.88
Estimated glomerular filtration rate (ml/min)	—	0.40
Serum cholinesterase (IU/L)	—	0.50

[a]Natural log transformations of the BNP and CRP levels were used for analysis.

The strengths of this study include highly reliable data, with up to 91% of availability among all participants, as well as novel parameters being assessed for an epidemiological study, such as central blood pressure, AI, CAVI, ABI, BNP, and hypersensitive-CRP. However, there are several limitations to this study. First, several parameters did not show statistical significance because of a low prevalence. Although a history of stroke, for example, has been previously reported as a correlate of nocturnal voiding, only 39 individuals (0.7%) in this study suffered from the disease. Second, our study could not accurately link the correlates of nocturnal voiding to potential mechanisms of nocturnal voiding, which included decreased bladder capacity, increased urine production during the night, and sleep problems. Third, care should be taken to interpret the observations, because our study did not demonstrate risk factors of nocturnal voiding but correlates of nocturnal voiding instead. A longitudinal prospective study is warranted to reveal the risk factors and the consequences of nocturnal voiding. Fourth, several factors that may affect nocturnal voiding, such as history of pelvic organ surgery, were lacking in the analyses in this study. Finally, it is unknown whether similar results are observed in communities outside Japan or in other races, and whether these results are true in nocturic patients under the care of doctors. Despite these limitations, our observations are clinically important since they suggest that clinical and preclinical heart failure represented by high serum BNP levels might be a potential cause or marker of nocturnal voiding.

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Meta-analysis identifies nine new loci associated with rheumatoid arthritis in the Japanese population

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Rheumatoid arthritis is a common autoimmune disease characterized by chronic inflammation. We report a meta-analysis of genome-wide association studies (GWAS) in a Japanese population including 4,074 individuals with rheumatoid arthritis (cases) and 16,891 controls, followed by a replication in 5,277 rheumatoid arthritis cases and 21,684 controls. Our study identified nine loci newly associated with rheumatoid arthritis at a threshold of $P < 5.0 \times 10^{-8}$, including *B3GNT2*, *ANXA3*, *CSF2*, *CD83*, *NFKBIE*, *ARID5B*, *PDE2A-ARAP1*, *PLD4* and *PTPN2*. *ANXA3* was also associated with susceptibility to systemic lupus erythematosus ($P = 0.0040$), and *B3GNT2* and *ARID5B* were associated with Graves' disease ($P = 3.5 \times 10^{-4}$ and 2.9×10^{-4}, respectively). We conducted a multi-ancestry comparative analysis with a previous meta-analysis in individuals of European descent (5,539 rheumatoid arthritis cases and 20,169 controls). This provided evidence of shared genetic risks of rheumatoid arthritis between the populations.

Rheumatoid arthritis is a complex autoimmune disease characterized by inflammation and the destruction of synovial joints and affects up to 1% of the population worldwide. To date, more than 35 rheumatoid arthritis susceptibility loci, including *HLA-DRB1*, *PTPN22*, *PADI4*, *STAT4*, *TNFAIP3* and *CCR6*, among others, have been identified by GWAS in multiple populations[1–12] and by several meta-analyses of the original GWAS[13–16]. In particular, each meta-analysis of these GWAS uncovered a number of loci that were not identified in the single GWAS, leading to recognition of the enormous power of the meta-analysis approach for detecting causal genes in disease. However, these previous meta-analyses have been performed solely in European populations[13–16] and not in Asian ones. As multi-ancestry studies on validated rheumatoid arthritis susceptibility loci showed the existence of both population-specific and shared genetic components of rheumatoid arthritis[10,17], additional studies in Asian populations might provide useful insight into the underlying genetic architecture of rheumatoid arthritis, which would otherwise be difficult to capture using the studies in a single population. Here, we report a meta-analysis of GWAS and a replication study for rheumatoid arthritis in a Japanese population that was conducted by the Genetics and Allied research in Rheumatic diseases NETworking (GARNET) consortium[10,12]. We subsequently performed a multi-ancestry comparative analysis that incorporated results from a previously conducted meta-analysis of individuals of European ancestry[15].

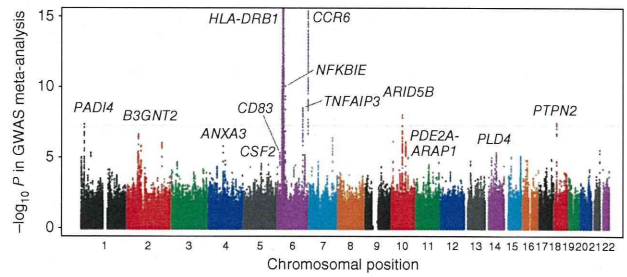

Figure 1 Manhattan plots of the GWAS meta-analysis for rheumatoid arthritis in the Japanese population. The genetic loci that satisfied the genome-wide significance threshold of $P < 5.0 \times 10^{-8}$ (gray line) in the meta-analysis or in the combined study of the meta-analysis and the replication study are presented. The *y* axis shows the $-\log_{10} P$ values of the SNPs in the meta-analysis. The SNPs for which the *P* values were smaller than 1.0×10^{-15} are indicated at the upper limit of the plot.

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Table 1 Results of the GWAS meta-analysis and the replication studies for rheumatoid arthritis

					Associations in Japanese								Associations in Europeans[c]			
					GWAS meta-analysis			Replication study		Combined study			GWAS meta-analysis			
					Allele	Allele 1 freq.							Allele 1 Freq.			
rsID[a]	Chr.	Position (bp)	Cytoband	Gene(s)	1/2	RA	Control	OR (95% CI)[b]	*P*	OR (95% CI)[b]	*P*	OR (95% CI)[b]	RA	Control	OR (95% CI)[b]	*P*
SNPs with significant associations ($P < 5.0 \times 10^{-8}$ in the combined study)																
rs11900673	2	62306165	2p15	*B3GNT2*	T/C	0.31	0.28	1.15 (1.08–1.21)	3.5×10^{-6}	1.09 (1.04–1.14)	6.0×10^{-4}	1.11 (1.07–1.15)	1.1×10^{-8}	0.13	0.13	1.05 (0.98–1.13)	0.17
rs2867461	4	79732239	4q21	*ANXA3*	A/G	0.46	0.44	1.13 (1.08–1.19)	4.7×10^{-6}	1.12 (1.08–1.17)	1.2×10^{-7}	1.13 (1.09–1.17)	1.2×10^{-12}	0.37	0.37	0.98 (0.92–1.04)	0.52
rs657075	5	131458017	5q31	*CSF2*	A/G	0.38	0.36	1.12 (1.06–1.18)	3.2×10^{-5}	1.11 (1.06–1.16)	3.8×10^{-6}	1.12 (1.08–1.15)	2.8×10^{-10}	0.10	0.10	1.04 (0.95–1.13)	0.37
rs12529514	6	14204637	6p23	*CD83*	C/T	0.16	0.14	1.19 (1.10–1.27)	6.8×10^{-6}	1.11 (1.05–1.18)	6.0×10^{-4}	1.14 (1.09–1.19)	2.0×10^{-8}	0.055	0.053	1.11 (0.99–1.24)	0.074
rs2233434	6	44340898	6p21.1	*NFKBIE*	G/A	0.24	0.21	1.23 (1.16–1.31)	9.2×10^{-11}	1.17 (1.11–1.23)	2.2×10^{-9}	1.19 (1.15–1.24)	5.8×10^{-19}	0.059	0.040	1.57 (1.11–2.21)	0.0099
rs10821944	10	63455095	10q21	*ARID5B*	G/T	0.39	0.36	1.17 (1.11–1.23)	1.0×10^{-8}	1.15 (1.10–1.20)	3.0×10^{-10}	1.16 (1.12–1.20)	5.5×10^{-18}	0.29	0.26	1.11 (1.05–1.17)	1.9×10^{-4}
rs3781913	11	72051144	11q13	*PDE2A-ARAP1*	T/G	0.71	0.69	1.11 (1.05–1.17)	3.2×10^{-4}	1.13 (1.08–1.18)	6.7×10^{-7}	1.12 (1.08–1.16)	5.8×10^{-10}	0.45	0.43	1.04 (0.99–1.09)	0.13
rs2841277	14	104462050	14q32	*PLD4*	T/C	0.72	0.69	1.11 (1.05–1.18)	2.8×10^{-4}	1.18 (1.13–1.24)	7.0×10^{-12}	1.15 (1.11–1.19)	1.9×10^{-14}	0.47	0.46	1.02 (0.96–1.09)	0.54
rs2847297	18	12787694	18p11	*PTPN2*	G/A	0.37	0.33	1.16 (1.11–1.23)	3.5×10^{-8}	1.06 (1.01–1.11)	0.013	1.10 (1.07–1.14)	2.2×10^{-8}	0.36	0.34	1.10 (1.05–1.15)	9.2×10^{-5}
SNPs with suggestive associations ($5.0 \times 10^{-8} \leq P < 5.0 \times 10^{-6}$ in the combined study)																	
rs4937362	11	127997949	11q24	*ETS1-FLI1*	T/C	0.71	0.68	1.13 (1.07–1.19)	2.0×10^{-5}	1.07 (1.02–1.12)	0.0061	1.09 (1.06–1.13)	7.5×10^{-7}	0.46	0.44	1.06 (1.01–1.11)	0.015
rs3783637	14	54417868	14q22	*GCH1*	C/T	0.76	0.74	1.13 (1.07–1.20)	6.5×10^{-5}	1.07 (1.02–1.13)	0.0062	1.10 (1.06–1.14)	2.0×10^{-6}	0.88	0.88	0.99 (0.88–1.11)	0.87
rs1957895	14	60978085	14q23	*PRKCH*	G/T	0.40	0.39	1.12 (1.06–1.18)	4.1×10^{-5}	1.07 (1.02–1.12)	0.0022	1.09 (1.05–1.13)	3.6×10^{-7}	0.093	0.089	1.01 (0.95–1.07)	0.73
rs6496667	15	88694672	15q26	*ZNF774*	A/C	0.38	0.35	1.13 (1.07–1.19)	4.7×10^{-5}	1.07 (1.02–1.11)	0.0050	1.09 (1.05–1.13)	1.4×10^{-6}	0.21	0.20	1.07 (1.01–1.13)	0.031
rs7404928	16	23796341	16p12	*PRKCB1*	T/C	0.65	0.62	1.13 (1.07–1.19)	1.5×10^{-5}	1.05 (1.01–1.10)	0.026	1.08 (1.05–1.12)	4.0×10^{-6}	0.75	0.75	1.01 (0.94–1.09)	0.79
rs2280381	16	84576134	16q24	*IRF8*	T/C	0.86	0.84	1.16 (1.08–1.25)	1.0×10^{-4}	1.09 (1.03–1.15)	0.0049	1.12 (1.07–1.17)	2.4×10^{-6}	0.62	0.60	1.05 (0.99–1.11)	0.081
SNPs in previously reported rheumatoid arthritis susceptibility loci ($P < 5.0 \times 10^{-8}$ in the GWAS)																	
rs766449	1	17547439	1p36	*PADI4*	T/C	0.44	0.40	1.17 (1.11–1.24)	4.6×10^{-8}	–	–	–	–	0.38	0.37	1.09 (1.03–1.05)	0.0022
rs2157337	6	32609122	6p21.3	*HLA-DRB1*	C/T	0.59	0.44	1.99 (1.88–2.11)	2.6×10^{-118}	–	–	–	–	0.69	0.46	2.50 (2.39–2.62)	$< 1.0 \times 10^{-300}$
rs6932056	6	138284130	6q23	*TNFAIP3*	C/T	0.092	0.073	1.35 (1.23–1.49)	3.2×10^{-9}	–	–	–	–	0.044	0.034	1.41 (1.24–1.60)	1.3×10^{-7}
rs1571878	6	167460832	6q27	*CCR6*	C/T	0.54	0.48	1.31 (1.24–1.39)	3.2×10^{-19}	–	–	–	–	0.47	0.43	1.13 (1.08–1.19)	5.9×10^{-7}

Chr., chromosome; Freq., frequency; RA, rheumatoid arthritis; OR, odds ratio; CI, confidence interval.
[a]SNPs with $P < 5.0 \times 10^{-6}$ in the combined study of the GWAS meta-analysis and the replication study or SNPs with $P < 5.0 \times 10^{-8}$ in the GWAS meta-analysis are annotated according to forward strand and NCBI Build 36.3. Full results of the replication study are provided in **Supplementary Table 3**. [b]Odds ratio of allele 1. [c]Associations in the previous meta-analysis in European populations[15].

The meta-analysis included 4,074 rheumatoid arthritis cases (with 81.4% and 80.4% of the subjects being positive for antibody to cyclic citrullinated peptide (anti-CCP) and rheumatoid factor, respectively) and 16,891 controls from three GWAS of Japanese subjects (from the BioBank Japan Project[10,18], Kyoto University[12] and the Institute of Rheumatology Rheumatoid Arthritis (IORRA)[19]; **Supplementary Table 1**). After the application of stringent quality control criteria, including principal-component analysis (PCA; **Supplementary Fig. 1**) for each GWAS, the meta-analysis was conducted by evaluating ~2.0 million autosomal SNPs with minor allele frequencies (MAFs) ≥0.01, which were obtained through whole-genome imputation of genotypes on the basis of the HapMap Phase 2 East Asian panels (Japanese in Tokyo (JPT) and Han Chinese in Beijing (CHB)). The inflation factor of the test statistics in the meta-analysis λ_{GC} was as low as 1.036, suggesting no substantial effects of population structure (**Supplementary Table 2**). The quantile-quantile plot of *P* values showed a marked discrepancy in the values in its tail from those anticipated under the null hypothesis that there is no association—even after removal of the SNPs located in the human leukocyte antigen (HLA) region, the major rheumatoid arthritis susceptibility locus—thereby showing the presence of significant associations in the meta-analysis (**Supplementary Fig. 2**).

We identified seven loci in the current meta-analysis that satisfied the genome-wide significance threshold of $P < 5.0 \times 10^{-8}$. These included previously known rheumatoid arthritis susceptibility loci, such as *PADI4* at 1p36, *HLA-DRB1* at 6p21.3, *TNFAIP3* at 6q23 and *CCR6* at 6q27 (refs. 1,3,6,10,15) (the smallest $P = 2.6 \times 10^{-118}$ was found at the *HLA-DRB1* locus; **Fig. 1** and **Table 1**). To our knowledge, the other three loci identified, *NFKBIE* at 6p21.1, *ARID5B* at 10q21 and *PTPN2* at 18p11, are newly associated ($P = 9.2 \times 10^{-11}$, 1.0×10^{-8} and 3.5×10^{-8}, respectively).

To validate the associations identified in the meta-analysis, we conducted a replication study of two independent Japanese rheumatoid arthritis case-control cohorts (cohort 1: 3,830 rheumatoid arthritis cases and 17,920 controls, cohort 2: 1,447 rheumatoid arthritis cases and 3,764 controls; **Supplementary Table 1**). To increase the number of subjects and enhance statistical power, genotype data obtained from other GWAS projects conducted for non-autoimmune diseases in Japanese using Illumina platforms were used for the replication control panels. For each of the 46 loci that exhibited $P < 5.0 \times 10^{-4}$ in

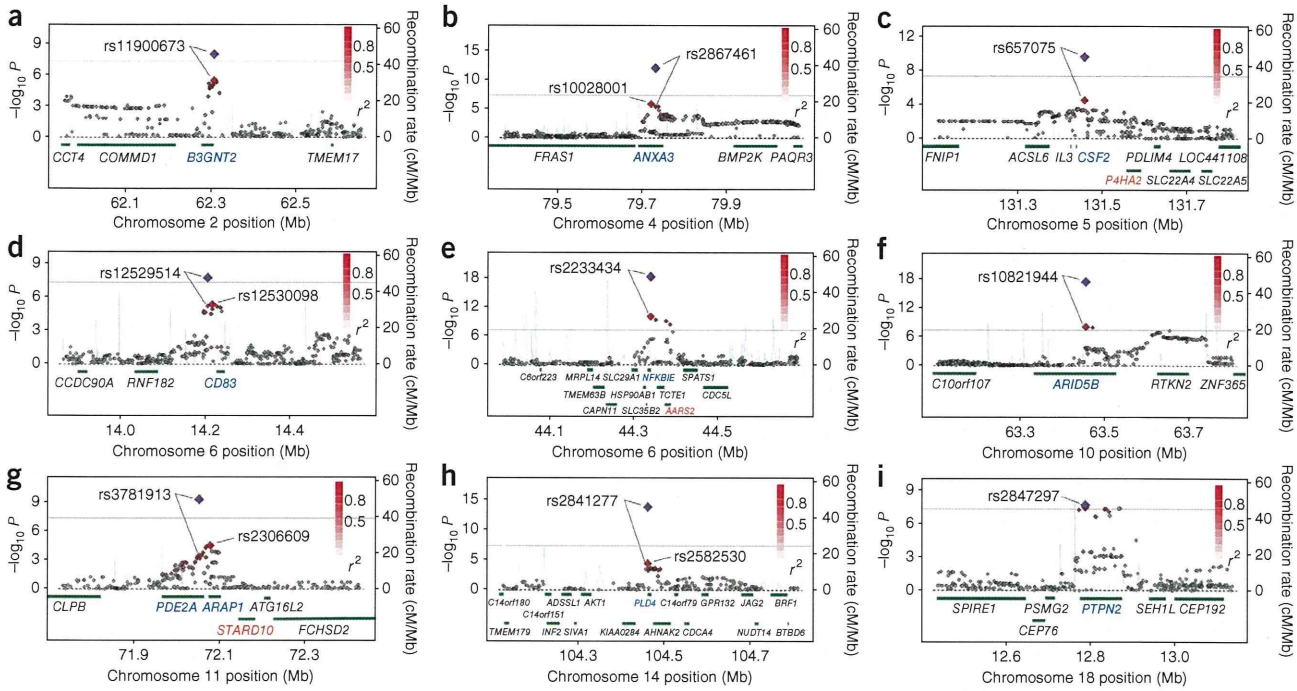

Figure 2 Regional plots of the loci newly associated with rheumatoid arthritis at the genome-wide significance threshold of $P < 5.0 \times 10^{-8}$ in the combined study of the meta-analysis and the replication study. (**a**–**i**) Regional plots are shown at *B3GNT2* (**a**), *ANXA3* (**b**), *CSF2* (**c**), *CD83* (**d**), *NFKBIE* (**e**), *ARID5B* (**f**), *PDE2A-ARAP1* (**g**), *PLD4* (**h**) and *PTPN2* (**i**). Diamonds represent the $-\log_{10} P$ values of the SNPs, and the red diamonds represent the $-\log_{10} P$ values of the SNPs in the meta-analysis. Red color for the smaller circles represents the r^2 value with the most significantly associated SNP (larger red circle). The purple circle represents the *P* value in the combined study. The blue line shows the recombination rates given by the HapMap Phase 2 east Asian populations (release 22). RefSeq genes at the loci are indicated below. Genes nearest to the marker SNPs at the loci are colored blue (**Supplementary Note**), and genes implicated in eQTL analysis are colored red (**Supplementary Table 4**). At 11q13, two genes (*PDE2A* and *ARAP1*) that are nearest to the SNP selected for the replication study and the most significant SNP in the meta-analysis are highlighted. The plots were drawn using SNP Annotation and Proxy Search (SNAP) version 2.2.

the meta-analysis and had not been reported as rheumatoid arthritis susceptibility loci[1–16], we selected a marker SNP for the replication study (Online Methods and **Supplementary Table 3**).

In the combined analyses of the meta-analysis and the replication study, including a total of 9,351 rheumatoid arthritis cases and 38,575 controls, we identified six newly associated loci, in addition to the *NFKBIE*, *ARID5B* and *PTPN2* loci, that satisfied the significance threshold of $P < 5.0 \times 10^{-8}$, including *B3GNT2* at 2p15, *ANXA3* at 4q21, *CSF2* at 5q31, *CD83* at 6p23, *PDE2A-ARAP1* at 11q13 and *PLD4* at 14q32 (**Figs. 1** and **2** and **Table 1**). Of these loci, *NFKBIE* had the smallest *P* value (5.8×10^{-19}). Although association with rheumatoid arthritis has been described for the *CSF2* and *PTPN2* loci[11,15,16,20,21], ours is the first report to our knowledge validating these associations with a threshold of $P < 5.0 \times 10^{-8}$. Suggestive associations were also observed in *ETS1-FLI1* at 11q24, *GCH1* at 14q22, *PRKCH* at 14q23, *ZNF774* at 15q26, *PRKCB1* at 16p12 and *IRF8* at 16q24 ($5.0 \times 10^{-8} \leq P < 5.0 \times 10^{-6}$). A summary of the genes in the newly associated loci and the results of *cis* expression quantitative trait locus (*cis* eQTL) analysis of the marker SNPs are provided (**Supplementary Table 4** and **Supplementary Note**).

Previous studies have reported associations of rheumatoid arthritis susceptibility loci with other autoimmune diseases[4,10,15,16]. Therefore, we assessed the association of these newly identified susceptibility loci with systemic lupus erythematosus (SLE) by examining the results of an SLE GWAS in the Japanese population (891 cases and 3,384 controls)[22] and in Graves' disease by genotyping 1,783 cases[10] (the controls from the SLE analysis were used for testing for Graves' disease). We observed significant associations of the *ANXA3* locus with SLE and of the *B3GNT2* and *ARID5B* loci with Graves' disease, which showed the same directional effects of the alleles as in rheumatoid arthritis ($P < 0.05/9 = 0.0056$, Bonferroni correction of the number of loci; **Supplementary Table 5**). It should be noted that relatively small sample sizes in the SLE and Graves' disease cohorts might yield limited statistical power, and further evaluations enrolling larger numbers of subjects would be desirable.

To highlight genetic backgrounds of rheumatoid arthritis that are common and divergent in different ancestry groups, we conducted a multi-ancestry comparative analysis of the present study in Japanese and a previous GWAS meta-analysis in Europeans that included 5,539 rheumatoid arthritis cases and 20,169 controls[15] (**Fig. 3a–c**). First, we compared associations in the reported[1–16] or newly identified rheumatoid arthritis susceptibility loci (**Fig. 3a** and **Supplementary Table 6**). Of the 46 rheumatoid arthritis risk variants evaluated, 6 were monomorphic in Japanese, and all were polymorphic in Europeans. We observed significant associations at 22 loci in Japanese and at 36 loci in Europeans (false discovery rate (FDR) < 0.05, $P < 0.0030$), with 14 loci being shared between the populations. Of the newly associated rheumatoid arthritis susceptibility loci identified in our Japanese meta-analysis, significant associations were also observed in the European meta-analysis at the *ARID5B* and *PTPN2* loci ($P = 1.9 \times 10^{-4}$ and 9.2×10^{-5}, respectively; **Table 1**). Significant positive correlation of odds ratios was observed between the studies ($r = 0.822$, $P = 8.1 \times 10^{-11}$; **Fig. 3b**), suggesting that a substantial proportion of genetic factors are shared between

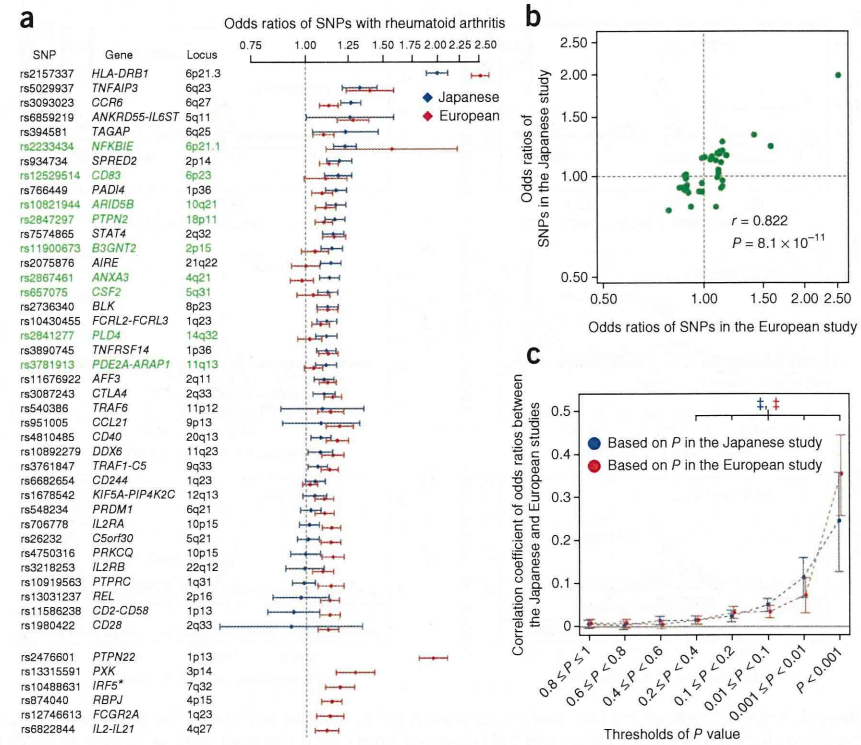

Figure 3 Overlap of the associations with rheumatoid arthritis between Japanese and European populations. (**a**) Forest plots of SNPs in the rheumatoid arthritis susceptibility loci (**Supplementary Table 6**). We selected the genetic loci that have been validated to be associated with rheumatoid arthritis susceptibility by showing associations in the reports of multiple cohorts or satisfying the genome-wide significant threshold ($P < 5.0 \times 10^{-8}$) in previous studies, including in the meta-analysis and replication phases[1–16]. For each of the loci, the most significant SNP among those reported in the previous or present study were selected[1–16]. SNPs in the newly identified rheumatoid arthritis susceptibility loci are colored green. Odds ratios and 95% confidence interval (CI) values are based on rheumatoid arthritis risk alleles, and the SNPs are ordered according to the odds ratios in the Japanese study. Several SNPs were monomorphic in the Japanese population. The odds ratios of these SNPs in the European study are presented below. The asterisk indicates that an association of another variant at the *IRF5* locus was reported in the Japanese population[24]. (**b**) Correlation of the odds ratios of the SNPs in the validated rheumatoid arthritis susceptibility loci between the two populations. SNPs that were polymorphic in both populations were used; odds ratios were based on the minor allele in the Japanese population. (**c**) Correlation of the odds ratios of the genome-wide SNPs, excluding the rheumatoid arthritis susceptibility loci. Correlations were evaluated for sets of SNPs stratified by the thresholds based on the meta-analysis *P* values in each population after pruning of the SNPs by LD ($r^2 < 0.3$). Correlation coefficient and 95% CI are indicated on the *y* axis. Significant correlation of the odds ratios was observed (‡, $P < 0.005$), even for the SNPs that showed moderate associations with rheumatoid arthritis (meta-analysis $P < 0.4$ in each population).

the two ancestry groups[17]. When the rheumatoid arthritis cases of the Japanese GWAS meta-analysis were stratified into anti-CCP–positive or rheumatoid factor–positive cases ($n = 3{,}209$) and controls ($n = 16{,}891$), similar results were observed (data not shown). Nevertheless, most of the SNPs assessed here are not necessarily causal variants, and further fine mapping of the loci is warranted to precisely evaluate the shared genetic predisposition between the populations.

Next, we compared regional associations within each of the loci and identified unique patterns in the *ARID5B* locus at 10q21 (**Supplementary Fig. 3**). In Japanese, three peaks of association were observed ($P = 1.0 \times 10^{-8}$ at rs10821944, $P = 5.7 \times 10^{-8}$ at rs10740069 and $P = 8.5 \times 10^{-6}$ at rs224311). These three variants were in weak linkage disequilibrium (LD) in Japanese ($r^2 < 0.10$), indicating independent associations with each of the other SNPs that satisfied a region-wide significance threshold of $P < 3.5 \times 10^{-5}$ (conditional $P = 4.3 \times 10^{-6}$, 1.7×10^{-5} and 1.8×10^{-5}, respectively) (**Supplementary Fig. 3**). In contrast, there was only one peak of association in Europeans ($P = 1.2 \times 10^{-6}$ at rs12764378; $r^2 = 0.59$ with rs10821944 in Europeans), and no additional association was observed in conditional analysis with rs12764378 (the smallest conditional $P = 2.2 \times 10^{-4}$), suggesting that the number of independent associations may be different at this locus in the two populations.

Finally, we conducted polygenic assessment for common variants showing modest associations to rheumatoid arthritis (those not meeting the genome-wide association threshold). This approach has been recognized to be a means to explain a substantial proportion of genetic risk[23]. For the SNPs that were shared between the two meta-analyses but not included in the validated rheumatoid arthritis susceptibility loci, we adopted LD pruning of the SNPs ($r^2 < 0.3$). We then evaluated the correlation of odds ratios of the SNPs between the two meta-analyses and observed a significant positive correlation ($r = 0.023$, $P < 1.0 \times 10^{-300}$). When the SNPs were stratified according to the *P* values in each meta-analysis, significant positive correlations of odds ratios were observed for the SNPs, even for those showing modest association ($P < 0.4$ in the meta-analysis of Japanese or Europeans; $r = 0.014$–0.36 for each *P* value range, $P < 0.005$ for each correlation test) (**Fig. 3c**). Correlations (r) of odds ratios observed herein suggest substantial overlap of the genetic risk of rheumatoid arthritis between the two populations, not only in the validated rheumatoid arthritis susceptibility loci but also at the loci showing nonsignificant associations. This suggests the usefulness of a meta-analysis approach involving multiple ancestry groups in identifying additional susceptibility loci.

In summary, we identified multiple new loci associated with rheumatoid arthritis through a large-scale meta-analysis of GWAS in Japanese. Multi-ancestry comparative analysis provided evidence of significant overlap in the genetic risks of rheumatoid arthritis between Japanese and Europeans. Thus, findings from the present study should contribute to the further understanding of the etiology of rheumatoid arthritis.

URLs. GARNET consortium, http://www.twmu.ac.jp/IOR/garnet/home.html; The BioBank Japan Project (in Japanese), http://biobankjp.org/; International HapMap Project, http://www.hapmap.org/; PLINK, http://pngu.mgh.harvard.edu/~purcell/plink/; EIGENSTRAT, http://genepath.med.harvard.edu/~reich/Software.htm; MACH and mach2dat, http://www.sph.umich.edu/csg/abecasis/MACH/index.

html; R statistical software, http://cran.r-project.org/; SNAP, http://www.broadinstitute.org/mpg/snap/index.php; NCBI GEO database, http://www.ncbi.nlm.nih.gov/geo/.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

Y. Okada, C.T., K.I., Y. Kochi and K.O. designed the study and drafted the manuscript. Y. Okada, C.T., K.I., T.K., H.O., N.N., M.T., M.L., K. Tokunaga and M.K. managed genotyping and manipulation of GWAS data. Y. Okada, Y. Kochi, C.T. and K.I. managed genotyping of replication cohorts. Y. Okada, T.K., H.O., E.A.S., A. Takahashi and R.Y. performed statistical analysis. Y. Kochi, A.S., K. Myouzen, T. Sawada, Y. Nishoka, M.Y., T. Matsubara, S.W., R.T. and S.T. collected samples and managed phenotype data for the rheumatoid arthritis cohorts from the BioBank Japan Project and CGM, RIKEN. C.T., K.O., T.K., M.T., K. Takasugi, K.S., A.M., S.H., K. Matsuo, H. Tanaka, K. Tajima and M.L. collected samples and managed phenotype data for the rheumatoid arthritis cohorts from Kyoto University. K.I., T. Suzuki, T.I., Y. Kawamura, H. Tanii, Y. Okazaki and T. Sakaki collected samples and managed phenotype data for the rheumatoid arthritis cohorts from IORRA. Y. Kochi managed the data for the SLE and Graves' disease cohorts. A.S., C.T. and K.I. analyzed the sera of subjects with rheumatoid arthritis. E.A.S., F.A.S.K., P.K.G., J.W., K.A.S., L.P. and R.M.P. managed the data for the rheumatoid arthritis cohorts in European populations. A. Taniguchi, A. Takahashi, K. Tokunaga, M.K., Y. Nakamura, N.K., T. Minori, R.M.P., H.Y., S.M., R.Y., F.M. and K.Y. supervised the overall study.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Subjects. The Japanese participants in the meta-analysis (4,074 rheumatoid arthritis cases and 16,891 controls) and the replication study (5,277 rheumatoid arthritis cases and 21,684 controls) were obtained through the collaborations of the GARNET consortium (**Supplementary Table 1**)[10,12]. The meta-analysis was conducted on three independent GWAS (from the BioBank Japan Project[18] with 2,414 rheumatoid arthritis cases and 14,245 controls[10], Kyoto University with 1,237 rheumatoid arthritis cases and 2,087 controls[12] and IORRA[19] with 423 rheumatoid arthritis cases and 559 controls). The replication study consisted of two independent cohorts (cohort 1 included 3,830 rheumatoid arthritis cases and 17,920 controls, and cohort 2 included 1,447 rheumatoid arthritis cases and 3,764 controls). We employed a case-control cohort of SLE (891 cases and 3,384 controls)[22] and 1,783 cases with Graves' disease[10]. Details of 5,539 rheumatoid arthritis cases and 20,169 controls included in the meta-analysis in European populations were described elsewhere[15]. All participants provided written informed consent for participation in the study, as approved by the ethical committees of the institutional review boards. Detailed descriptions of the participating subjects are provided (**Supplementary Note**).

Genotyping and quality control in the GWAS. Genotyping platforms and quality control criteria for the GWAS, including cutoff values for sample call rates, SNP call rates, MAF and Hardy-Weinberg *P* values, are given (**Supplementary Table 2**). For the subjects enrolled in each of three GWAS, we excluded closely related subjects with first- or second-degree kinship, which was estimated using PLINK version 1.06 (see URLs). We also excluded the subjects determined to be ancestry outliers from East Asian populations using PCA performed by EIGENSTRAT version 2.0 (see URLs) along with HapMap Phase 2 panels (release 24; **Supplementary Fig. 1**). Genotype imputation was performed on the basis of the HapMap Phase 2 East Asian populations, using MACH version 1.0.16 (see URLs) in a two-step procedure as described elsewhere[25]. We excluded imputed SNPs with MAF < 0.01 or *Rsq* < 0.5 from each of the GWAS. Associations of the SNPs with rheumatoid arthritis were assessed by logistic regression models assuming additive effects of the allele dosages of the SNPs using mach2dat software (see URLs).

Meta-analysis. We included 1,948,139 autosomal SNPs that satisfied quality control criteria in all three GWAS (**Supplementary Table 2**). SNP information was based on a forward strand of the NCBI build 36.3 reference sequence. The meta-analysis was performed using an inverse variance method assuming a fixed-effects model from the study-specific effect sizes (logarithm of odds ratio) and the standard errors of the coded alleles of the SNPs determined with the Java source code implemented by the authors[25]. Genomic control corrections[26] were carried out on test statistics of the GWAS using the study-specific inflation factor (λ_{GC}) and was applied or reapplied to the results of our current meta-analysis (**Supplementary Fig. 2**).

Replication study. We selected a SNP for the replication study from each of the loci that exhibited $P < 5.0 \times 10^{-4}$ in the meta-analysis that had not previously been reported as rheumatoid arthritis susceptibility loci[1–16] (**Supplementary Table 3**). For control subjects, we used genotype data obtained from additional GWAS for non-autoimmune diseases or healthy controls, genotyped using Illumina HumanHap550 BeadChips or HumanHap610-Quad BeadChips, and the cases for rheumatoid arthritis and Graves' disease were genotyped with the TaqMan genotyping system (Applied Biosystems; **Supplementary Table 1**). Selection of the SNP was conducted according to the following criteria: if the SNP with the most significant association in the locus was genotyped in the replication control panel, then that SNP was selected; otherwise, a tag SNP in the replication control panel with the strongest LD was selected (mean $r^2 = 0.89$). For the three SNPs that yielded low call rates (<90%), we alternatively selected proxy SNPs with the second strongest LD. As a result, average genotyping call rates of the SNPs were 99.9% and 99.0% for the controls and cases, respectively. We then evaluated concordance rates between the assayed genotypes by applying these two different methods to samples from 376 subjects who were randomly selected. This procedure yielded high concordance rates of ≥99.9%. Associations of the SNPs were evaluated using logistic regression assuming an additive-effects model of genotypes in R statistical software version 2.11.0 (see URLs). The combined study of the meta-analysis and replication study was performed using an inverse variance method assuming a fixed-effects model[25].

***Cis* eQTL analysis.** For each marker SNP of the newly identified rheumatoid arthritis susceptibility locus, correlations between SNP genotypes and expression levels of genes located 300 kb upstream or downstream of the SNP measured in B-lymphoblastoid cell lines (GSE6536) were evaluated using data from the HapMap Phase 2 east Asian populations[27].

Multi-ancestry analysis of the meta-analyses in Japanese and Europeans. We evaluated the associations of the variants in the validated rheumatoid arthritis susceptibility loci by comparing the results from the current meta-analysis in Japanese with those from a previous meta-analysis in Europeans[15]. We assessed two variants in the *IRF5* locus, where different causal variants were identified in the two populations[24]. For the conditional analysis of the regional associations in the *ARID5B* locus (**Supplementary Fig. 3**), we repeated the meta-analysis at that locus by incorporating genotypes of the referenced SNP(s) as additional covariate(s). For comparison of the odds ratios of the SNPs, we first selected SNPs that were shared between the meta-analyses in Japanese and Europeans. Next, we removed the SNPs located more than 1 Mb away from each of the marker SNPs in the validated rheumatoid arthritis susceptibility loci, except for in the HLA region, where we removed the SNPs located between 24,000,000 bp to 36,000,000 bp on chromosome 6 because of the existence of long-range haplotypes with rheumatoid arthritis susceptibility in this region[28]. LD pruning of the SNPs was conducted for the SNP pairs that were in LD ($r^2 \geq 0.3$) in both HapMap Phase 2 East Asian and Utah residents of Northern and Western European ancestry (CEU) populations (release 24). Correlations of the odds ratios were evaluated using R statistical software version 2.11.0.

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Vascular Endothelial Growth Factor Gene Polymorphisms and Choroidal Neovascularization in Highly Myopic Eyes

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Purpose. To investigate a potential association between VEGF gene polymorphisms and the occurrence and/or the size of choroidal neovascularization (CNV) in highly myopic eyes.

Methods. In the case-control study for CNV occurrence, 327 highly myopic Japanese patients were enrolled. One hundred and eighty-four patients had CNV in at least one eye, and 143 did not have CNV in either eye. Of the 184 patients with CNV, 83 patients were used to evaluate an association with CNV size, and an additional 76 patients with CNV were used to confirm the association. We genotyped four tag single nucleotide polymorphisms (SNPs) and four functional SNPs previously reported to be correlated with VEGF gene expression to evaluate the associations of these eight SNPs with CNV occurrence and size. To confirm the association between CNV size and VEGF gene polymorphism, the associated SNP was genotyped in 76 additional patients with myopic CNV.

Results. There was no significant association between the occurrence of myopic CNV and the SNPs in the VEGF gene ($P > 0.16$). Of the eight SNPs evaluated, however, rs2010963 showed significant association with CNV area ($P = 0.0047$). This association was successfully replicated in the additional 76 eyes with myopic CNV, and pooled analysis revealed significant association of rs2010963 with CNV size ($P = 0.00078$).

Conclusions. VEGF gene polymorphisms were not associated with CNV occurrence in highly myopic eyes but were significantly associated with the size of CNV, suggesting roles in the growth rather than the emergence of CNV. (*Invest Ophthalmol Vis Sci.* 2012;53:2349–2353) DOI:10.1167/iovs.11-9405

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Myopia is one of the most common ocular disorders worldwide. The prevalence of myopia is much higher in Asian populations, with a reported incidence of roughly 40% in the Japanese and Chinese population and 25% in Caucasians.[1–3] Pathological myopia, also called high myopia, is defined as a spherical equivalent refractive error of at least −6 diopters or an axial length ≥26.5 mm. Myopic axial length elongation can lead to chorioretinal atrophy and choroidal neovascularization (CNV), which is the most vision-threatening complication in highly myopic eyes.[4] Since the long-term visual outcomes of myopic CNV are extremely poor,[5] it is critical to determine in which highly myopic patients CNV will occur. CNV usually occurs in young adults with high myopia in the fourth and fifth decades of life. However, many eyes with high myopia do not have CNV even after 60 years of age. Furthermore, the size of the CNV seriously affects the visual prognosis because it determines the size of the scotoma, and some smaller CNVs can regress without treatment.[6] Since it is difficult to prevent the development of myopia, it is important to investigate the mechanisms underlying CNV occurrence and growth in myopic eyes; this may lead to the prevention of CNV development and the subsequent visual disturbance.

Genetic backgrounds may affect the development of high myopia; recently, we have determined a susceptible locus for pathological myopia using a genome-wide association study (GWAS).[7] Furthermore, recent GWASs reveal that myopia susceptibility loci exist in chromosome 15.[8–10] The occurrence of CNV in highly myopic eyes might also depend on genetic variations. Thus far, however, few studies have investigated the genetic background of patients with CNV in highly myopic eyes.

Since anti-VEGF treatment has been developed for neovascular AMD, it has become a popular treatment for ocular neovascularization. Anti-VEGF drugs have been shown to be effective in treating CNV secondary to high myopia.[11–13] In contrast to neovascular AMD, myopic CNV is easily inactivated with anti-VEGF treatment. In this study, we evaluated the associations between VEGF gene polymorphisms and CNV development in highly myopic eyes in Japanese patients.

Methods

This study was performed in accordance with the tenets of the Declaration of Helsinki. The Institutional Review Board/Ethics Committee of each institution approved the study protocols. All patients were fully informed of the study purpose and procedures, and written consent was obtained from each patient. For the case-control study of CNV occurrence, 327 highly myopic, unrelated Japanese patients with axial lengths of >26.0 mm in both eyes and who were ≥60 years of age were recruited from Kyoto University Hospital and Tokyo Medical and

TABLE 1. Characteristics of the Study Population

	With CNV	Without CNV	P Value
Number	184	143	
Mean age ± SD (years)	69.97 ± 6.35	69.23 ± 6.74	0.52*
Axial length ± SD (mm)			
Right	28.97 ± 1.72	29.11 ± 1.72	0.49*
Left	28.75 ± 1.72	28.84 ± 1.86	0.68*
Sex (male/female)	32/152	58/85	3.27×10^{-6}†

* Unpaired *t*-test.
† χ^2 test.

Dental University Hospital. The number of patients with macular CNV in at least one eye was 184, and the number of patients without macular CNV in either eye was 143 (Table 1). All patients underwent detailed ophthalmologic examinations, including dilated indirect and contact lens slit-lamp biomicroscopy, automatic objective refraction, measurement of the axial length by A-scan ultrasound (UD-6000; Tomey, Nagoya, Japan) or partial coherence interferometry (IOLMaster; Carl Zeiss Meditec, Dublin, CA), color fundus photography, optical coherence tomography, and fluorescein angiography. Individuals with a history of ocular surgery, with the exception of cataract surgery, were excluded from the study. Patients with secondary choroidal neovascular diseases, such as angioid streaks, presumed ocular histoplasmosis syndrome, and ocular trauma, were also excluded.

Of the 184 patients with myopic CNV, 83 patients underwent angiography with HRA2 (Heidelberg Engineering, Heidelberg, Germany) in Kyoto University Hospital. To evaluate the association between VEGF gene polymorphisms and CNV size, the area of CNV (mm^2) in these 83 patients was measured with the HRA-2 software. An additional 76 patients with myopic CNV were enrolled from Kyoto University Hospital to confirm the aforementioned associations. The average age of these patients was 63.8 ± 12.6 years, and the average axial length was 30.1 ± 1.1 mm.

For selecting tag single nucleotide polymorphisms (SNPs), we used the public dbSNP database build 126 (NCBI build 36.1) and HapMap database phase 2, release 22,[35] to extract the relevant sequencing information for the *VEGFA* gene and the genotyping information for the SNPs. A set of four tagging VEGF SNPs were selected for investigation: two SNPs on the promoter region, named rs699946 and rs699947, and two intronic SNPs, rs3025033 and rs3025035. This set of four tagging SNPs provided 100% coverage for all 14 common HapMap SNPs within a 26.3 kb region (16.3 kb gene length; 10 kb upstream) spanning the VEGF gene on chromosome 6 (r^2 threshold of 0.95). Furthermore, we evaluated four functional SNPs (rs1570360, rs2010963, rs833061, and rs3025039). Since these SNPs have been shown to affect VEGF expression,[14–17] many studies have evaluated the association of these SNPs with various diseases such as AMD, diabetic retinopathy, Behçet's disease, Alzheimer's disease, and diabetes.[18–26]

Genomic DNA was prepared from peripheral blood by a DNA extraction kit (QuickGene-610L; Fujifilm, Minato, Tokyo, Japan). VEGF-tagged SNPs (rs699946, rs699947, rs3025033, and rs3025035) and functional SNPs (rs1570360, rs2010963, rs833061, and rs3025039) were genotyped by a Taqman SNP assay with the ABI PRISM 7700 system (Applied Biosystems, Foster, CA). Deviations in genotype distributions from the Hardy-Weinberg equilibrium (HWE) were assessed with the HWE exact test. A χ^2 test for trend or its exact counterpart was used to compare the genotype distributions of the two groups. To adjust for age and sex, we performed logistic regression analysis. Mean age and axial length were compared using unpaired *t*-test or ANOVA, and sex ratio was compared with the χ^2 test. The associations between genotype and CNV size were evaluated using the Jonckheere-Terpstra trend test. *P* values of less than 0.05 were considered statistically significant.

RESULTS

The demographics of the study population are shown in Table 1; there was no significant difference between patients with CNV and patients without CNV with respect to either age or axial length. The mean age of each group was 70.0 ± 6.4 years and 69.2 ± 6.7 years, respectively ($P = 0.52$). However, CNV is more predominant in women compared with men ($P = 3.27 \times 10^{-6}$) with an odds ratio (OR) of 3.24 (95% confidence interval [CI] = 2.27–4.64).

The genotype counts, associations, and ORs for the eight SNPs are shown in Table 2. The genotype distributions were not significantly different between patients with CNV and patients without CNV (nominal $P > 0.16$). Evaluation of the associations in a recessive model and a dominant model also showed no associations ($P > 0.10$). Even when adjusted for age and sex, the genotype distributions were not significantly different ($P > 0.10$).

In addition, we performed subset analysis for patients aged 70 years or older. In our cohort, 86 patients with CNV and 63 patients without CNV were ≥70 years of age. Associations between the eight SNPs with the occurrence of CNV were not statistically significant ($P > 0.17$).

Of the 184 patients with myopic CNV, the area of CNV was measured in 83 patients who underwent angiography with HRA2 in Kyoto University Hospital. The genotype distribution of rs2010963 was significantly correlated with CNV area ($P = 0.0047$), while the other seven SNPs did not show significant associations with CNV area (Fig. 1). The size of CNV was largest (1.71 ± 1.29 mm^2) in patients with a CC genotype of rs2010963, intermediate (0.98 ± 0.84 mm^2) with a CG genotype, and smallest (0.78 ± 0.78 mm^2) with a GG genotype. There was no significant difference in axial length, age of patients, or male/female ratio among the three

TABLE 2. Genotype Counts, Associations, and Odds Ratios for VEGF SNPs

		CNV (+)			CNV (−)				Age- and Sex-Adjusted	
SNP	Genotype	Genotype Count	MAF	HWE *P*	Genotype Count	MAF	HWE *P*	Nominal *P*	*P*	OR (95% CI)
rs699946	AA/AG/GG	64/82/33	G, 0.41	0.399	40/73/23	G, 0.44	0.250	0.543	0.10	0.80 (0.62–1.04)
rs699947	AA/AC/CC	22/77/85	A, 0.33	0.477	17/60/63	A, 0.34	0.626	0.856	0.68	0.93 (0.66–1.31)
rs3025033	AA/AG/GG	125/53/4	G, 0.17	0.286	90/44/8	G, 0.21	0.151	0.160	0.60	0.94 (0.73–1.20)
rs3025035	CC/CT/TT	90/71/17	T, 0.29	0.391	79/49/12	T, 0.26	0.200	0.355	0.34	1.13 (0.88–1.44)
rs1570360	AA/AG/GG	11/42/130	A, 0.17	0.005	8/32/102	A, 0.17	0.020	0.858	0.79	0.94 (0.60–1.47)
rs2010963	CC/GC/GG	34/84/62	C, 0.42	0.547	23/73/42	C, 0.43	0.348	0.820	0.42	0.88 (0.65–1.20)
rs833061	CC/CT/TT	22/75/82	C, 0.33	0.451	17/60/66	C, 0.33	0.554	0.922	0.69	0.93 (0.66–1.31)
rs3025039	CC/CT/TT	116/56/5	T, 0.19	0.402	87/45/8	T, 0.22	0.298	0.328	0.81	0.97 (0.76–1.24)

MAF, minor allele frequency.