25. Cheung CY, Tso AW, Cheung BM et al (2010) Obesity susceptibility genetic variants identified from recent genome-wide association studies: implications in a Chinese population. J Clin Endocrinol Metab 95:1395–1403
26. Tsuchihashi-Makaya M, Serizawa M, Yanai K et al (2009) Gene–environmental interaction regarding alcohol-metabolizing enzymes in the Japanese general population. Hypertens Res 32:207–213
27. Nanri A, Yoshida D, Yamaji T, Mizoue T, Takayanagi R, Kono S (2008) Dietary patterns and C-reactive protein in Japanese men and women. Am J Clin Nutr 87:1488–1496
28. Takeuchi F, Serizawa M, Yamamoto K et al (2009) Confirmation of multiple risk loci and genetic impacts by a genome-wide association study of type 2 diabetes in the Japanese population. Diabetes 58:1690–1699
29. Purcell S, Neale B, Todd-Brown K et al (2007) PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet 81:559–575
30. Schousboe K, Willemsen G, Kyvik KO et al (2003) Sex differences in heritability of BMI: a comparative study of results from twin studies in eight countries. Twin Res 6:409–421
31. Cochran WG (1954) The combination of estimates from different experiments. Biometrics 10:101–129
32. Horikawa Y, Miyake K, Yasuda K et al (2008) Replication of genome-wide association studies of type 2 diabetes susceptibility in Japan. J Clin Endocrinol Metab 93:3136–3141
33. Omori S, Tanaka Y, Takahashi A et al (2008) Association of CDKAL1, IGF2BP2, CDKN2A/B, HHEX, SLC30A8, and KCNJ11 with susceptibility to type 2 diabetes in a Japanese population. Diabetes 57:791–795
34. Horikoshi M, Hara K, Ito C et al (2007) Variations in the HHEX gene are associated with increased risk of type 2 diabetes in the Japanese population. Diabetologia 50:2461–2466
35. Li X, Song F, Jiang H et al (2010) A genetic variation in the fat mass- and obesity-associated gene is associated with obesity and newly diagnosed type 2 diabetes in a Chinese population. Diabetes Metab Res Rev 26:128–132
36. Liu Y, Liu Z, Song Y et al (2010) Meta-analysis added power to identify variants in FTO associated with type 2 diabetes and obesity in the Asian population. Obesity (Silver Spring) 18:1619–1624
37. Ng MC, Park KS, Oh B et al (2008) Implication of genetic variants near TCF7L2, SLC30A8, HHEX, CDKAL1, CDKN2A/B, IGF2BP2, and FTO in type 2 diabetes and obesity in 6,719 Asians. Diabetes 57:2226–2233
38. Chang YC, Liu PH, Lee WJ et al (2008) Common variation in the fat mass and obesity-associated (FTO) gene confers risk of obesity and modulates BMI in the Chinese population. Diabetes 57:2245–2252
39. Yajnik CS, Janipalli CS, Bhaskar S et al (2009) FTO gene variants are strongly associated with type 2 diabetes in South Asian Indians. Diabetologia 52:247–252
40. Sanghera DK, Ortega L, Han S et al (2008) Impact of nine common type 2 diabetes risk polymorphisms in Asian Indian Sikhs: PPARG2 (Pro12Ala), IGF2BP2, TCF7L2 and FTO variants confer a significant risk. BMC Med Genet 9:59
41. Rong R, Hanson RL, Ortiz D et al (2009) Association analysis of variation in/near FTO, CDKAL1, SLC30A8, HHEX, EXT2, IGF2BP2, LOC387761, and CDKN2B with type 2 diabetes and related quantitative traits in Pima Indians. Diabetes 58:478–488
42. Renstrom F, Payne F, Nordstrom A et al (2009) Replication and extension of genome-wide association study results for obesity in 4923 adults from northern Sweden. Hum Mol Genet 18:1489–1496
43. Zollner S, Pritchard JK (2007) Overcoming the winner's curse: estimating penetrance parameters from case–control data. Am J Hum Genet 80:605–615
44. Fujita M, Ueno K, Hata A (2009) Effect of obesity on incidence of type 2 diabetes declines with age among Japanese women. Exp Biol Med (MayWood) 234:750–757
45. Sairenchi T, Iso H, Irie F, Fukasawa N, Ota H, Muto T (2008) Underweight as a predictor of diabetes in older adults: a large cohort study. Diabetes Care 31:583–584
46. Nakanishi S, Okubo M, Yoneda M, Jitsuiki K, Yamane K, Kohno N (2004) A comparison between Japanese-Americans living in Hawaii and Los Angeles and native Japanese: the impact of lifestyle westernization on diabetes mellitus. Biomed Pharmacother 58:571–577
47. WHO Expert Consultation (2004) Appropriate body-mass index for Asian populations and its implications for policy and intervention strategies. Lancet 363:157–163
48. Zobel DP, Andreasen CH, Grarup N et al (2009) Variants near MC4R are associated with obesity and influence obesity-related quantitative traits in a population of middle-aged people: studies of 14,940 Danes. Diabetes 58:757–764
49. Teramoto T, Sasaki J, Ueshima H et al (2007) Diagnostic criteria for dyslipidemia. Executive summary of Japan Atherosclerosis Society (JAS) guideline for diagnosis and prevention of atherosclerotic cardiovascular diseases for Japanese. J Atheroscler Thromb 14:155–158

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ORIGINAL ARTICLE
Molecular Cardiology

Confirmation of ALDH2 as a Major Locus of Drinking Behavior and of Its Variants Regulating Multiple Metabolic Phenotypes in a Japanese Population

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Background: Normative alcohol use (or drinking behavior) influences the risk of cardiovascular disease in a multi-faceted manner. To identify susceptibility gene variants for drinking behavior, a 2-staged genome-wide association study was performed in a Japanese population.

Methods and Results: In the stage-1 scan, 733 cases and 729 controls were genotyped with 456,827 SNP markers. The associated loci without redundancy of linkage disequilibrium were further examined in the stage-2 general population panel comprising 2,794 drinkers (≥once per week), 1,521 chance drinkers (<once per week), and 1,351 non-drinkers. Along with genome-wide exploration, we aimed to replicate the trait association of a candidate gene SNP previously reported (rs1229984 in *ADH1B*). A cluster of 12 SNPs on 12q24 were found to significantly ($P<5\times10^{-8}$) associate with drinking behavior in stage 1, among which rs671 (a Glu-to-Lys substitution at position 504) in the *ALDH2* gene showed the strongest association (odds ratio (OR)=0.16, $P=3.6\times10^{-211}$ in the joint analysis). The association was also replicated for rs1229984 (OR=1.20, $P<3.6\times10^{-4}$). Furthermore, *ALDH2* 504Lys was associated with several metabolic traits, eg, lower levels of high-density lipoprotein cholesterol and liver enzymes—AST, ALT, and γGTP—by interacting with alcohol intake.

Conclusions: Our results confirm *ALDH2* as a major locus regulating drinking behavior in the Japanese, indicating that the *ALDH2* 504Lys variant exerts pleiotropic effects on risk factors of cardiovascular disease among drinkers. (*Circ J* 2011; **75:** 911–918)

Key Words: Alcohol; *ALDH2*; Asians; Genetic susceptibility; Genome-wide association study

Alcohol consumption, a lifestyle factor prevalent globally, has been suggested to be relevant to atherosclerotic risk factors, such as hypertension, obesity, dyslipidemia, and diabetes.[1] Epidemiological studies have demonstrated that moderate alcohol intake lowers the risk of cardiovascular disease in a multi-faceted manner;[2,3] for example, mild to moderate alcohol consumption has a favorable influence on high-density lipoprotein cholesterol (HDL-C) level[2] and glucose metabolism,[4] whereas it causes hypertension[5] and hyper-triglyceridemia,[6] on the other hand.

Both environmental and genetic factors are assumed to contribute to inter-individual differences in alcohol consumption.[7] Here, alcohol drinking 'behavior' can be broadly dichotomized in alcoholism (or alcohol dependence) and normative alcohol use. As alcohol dependence constitutes a substantial health and economic burden, much effort has been directed to clarify its pathogenesis. In this line, a genome-wide association (GWA) study has been recently performed for alcohol dependence besides test of candidate genes (eg, those involved in GABAergic function) in populations of European

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Table 1. Clinical Characteristics of Study Participants

	GWA study panel	Amagasaki Study panel		
		Drinker	Chance drinker	Non-drinker
Number of subjects (M/F)	830/632	2,206/588	703/818	475/876
Age, year	66.3±8.0	49.8±11.7	44.3±12.9	51.5±12.4
BMI, kg/m²	23.5±3.3	23.2±2.9	22.7±3.4	22.7±3.4
AST, IU/L	NA	25.4±12.5	21.6±8.7	22.3±9.7
ALT, IU/L	24.4±22.1	26.2±19.5	22.6±18.9	21.6±16.5
γGTP, IU/L	34.6±63.1	38.0±47.6	18.4±19.6	16.6±13.8
Triglycerides, mg/dl	122.3±71.4	123.1±100.5	100.6±66.0	101.7±62.9
LDL-C, mg/dl	122.4±31.4	120.0±31.2	122.7±31.5	130.8±30.5
HDL-C, mg/dl	60.1±16.8	62.9±17.9	62.3±17.3	63.2±17.8
% of dyslipidemia treatment	57.5	2.7	2.7	4.4
Uric acid, mg/dl	5.4±1.3	5.7±1.4	4.9±1.3	4.7±1.2
% of gout treatment	6.1	2.3	1.0	0.3
FPG, mmol/L	5.38±0.62	5.5±1.1	5.2±0.9	5.3±1.1
% of diabetes treatment	30.8	6.5	4.6	5.2
SBP, mmHg	133.5±19.8	127.8±17.3	119.9±16.1	123.3±17.1
DBP, mmHg	76.6±11.9	78.4±10.8	72.8±10.4	74.6±10.6
% of hypertension treatment	47.8	11.8	6.1	9.3
Smoking behavior				
Non smoker, %	55.8	42.0	64.4	72.6
Past smoker, %	11.6	13.9	6.7	4.6
Current smoker, %	32.7	44.0	28.9	22.8
ALDH2 rs671 genotype				
%GG/%AG/%AA	56.0/37.6/6.4	72.7/27.1/0.2	55.8/42.8/1.5	23.1/52.7/24.3

Values are means±SD unless otherwise indicated.
In the Amagasaki Study panel, drinkers are defined as those who take alcoholic beverages ≥once a week, and chance drinkers are those who take alcoholic beverages <once a week.
GWA, genome-wide association; BMI, body mass index; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; FPG, fasting plasma glucose; SBP, systolic blood pressure; DBP, diastolic blood pressure.

descent.[8] From a public health viewpoint, however, we should give an equivalent degree of attention to normative alcohol use.

Ethanol acts in areas of the brain and on signaling cascades that are evolutionarily conserved among mammals. It has been shown in rodents that low level of responses to alcohol, which appear to increase the risk for lifetime alcohol dependence in humans, are genetically determined in part.[9] Rats selectively bred for alcohol preference and non-preference have been used for studying the behavioral and molecular basis of alcohol drinking.[10] Biological mechanisms that are assumed to serve to promote and maintain a high alcohol drinking behavior are: (1) increased potency of low-dose alcohol as a reinforcer; (2) weaker aversion to the pharmacological effects of moderate/high doses of alcohol; and (3) rapid induction of tolerance to the aversive effects of alcohol with repeated bouts of voluntary alcohol drinking.

Apart from several candidate genes involved in ethanol and acetaldehyde metabolism,[11–14] genetic susceptibility to normative alcohol use has not been widely explored. Therefore, in the present study, we performed a GWA study to identify susceptibility loci for alcohol drinking behavior in a Japanese population, followed by examination of their relevance to metabolic trait levels.[15]

Methods

Study Populations

We first performed GWA scan for alcohol drinking behavior using the genotype data on 1,462 Japanese samples (Table 1), which are part of our ongoing GWA study of cardiometabolic disorders among the Japanese as previously reported.[16] Then, we examined the association signals in a general population, known as the Amagasaki Study, as described elsewhere.[11] Briefly, to investigate lifestyle factors and genetic susceptibility to cardiovascular disease and its risk factor traits, we enrolled individuals who sought medical assessment from September 2002 to August 2003 at the Amagasaki Health Medical Foundation. The participants were included if they were over 18 years of age and had full clinical examination data, along with a completed questionnaire on their lifestyle. Among those enrolled in this prospective cohort study, 5,666 subjects (3,384 men/2,282 women) were used for the present association study.

Drinking behavior was assessed on the basis of the lifestyle questionnaire self-administered by the subjects. Alcohol consumption was classified into 2 categories: ever-drinkers (ie, ex-drinkers and current drinkers) and non-drinkers. Also, according to the current status and frequency of drinking, the subjects in the Amagasaki Study panel were categorized into 3 groups: non-drinker (n=1,351), chance drinker (less than once in a week, n=1,521) and drinker (equal to or more than once in a week, n=2,794). Since no strict distinction could be made by definition between chance drinkers and (habitual) drinkers, we arbitrarily defined habitual drinkers as above-mentioned in the present study, according to the national survey conducted in 2003 (http://www.e-healthnet.mhlw.go.jp/ (in Japanese, accessed on 9 Feb 2011). For chance drinkers and

drinkers, alcohol intake was self-reported as the usual amounts that were denoted in terms of servings of sake (1 gou (180 ml) of Japanese rice wine is considered equal to 22 g of ethanol). In addition, for part of drinkers (n=1,741), more detailed information was available on the type of alcoholic beverages consumed, the weekly frequency of alcohol consumption, and the usual amount of alcohol consumed per day; 1 gou of sake was defined as equivalent to 500 ml of beer, 2/3 gou of shochu, 2 single whiskeys, or 240 ml of wine. Based on the type and amount of beverage consumed, we calculated the total units (in gou) of ethanol for the weekly intake as a measure of alcohol intake in the quantitative analysis (**Figure S1**). The blood samples were collected during a visit to measure glucose, lipid, liver enzyme, and uric acid concentrations using standard techniques. Blood pressure was measured using an automatic sphygmomanometer in the sitting position after at least 5-min of rest. Smoking habits were classified into 3 categories: current smokers, past-smokers, and non-smokers.

All participants from these different studies provided written informed consent, and the local ethics committees approved the protocols.

SNP Genotyping and Quality Control

In the GWA scan, genotyping was performed with the Infinium HumanHap550 BeadArray (Illumina), which interrogated 555,352 SNPs (**Data S1**). Data cleaning and analysis were performed using PLINK software[17] as described elsewhere.[18] Population stratification was checked by multidimensional scaling analysis of the pair-wise distance between samples, measured over all SNPs (**Data S1**). The lambda value for the genomic control[19] was 1.01, indicating the absence of systematic confounding, such as population stratification, in the GWA study panel (**Data S1**).

After the GWA scan, 2 additional SNPs, rs671 in *ALDH2* and rs1229984 in *ADH1B*, were chosen for genotyping with the TaqMan assay (Applied Biosystems) as previously described[20] in the GWA study (stage 1) panel. Here, rs671 and rs1229984 were added to the GWA scan because of their positional (rs671 on 12q24) and physiological (*ALDH2* and *ADH1B*) candidacy. Although we had tested these 2 SNPs as part of 10 candidate SNP loci with relation to drinking behavior and the sensitivity to pressor effects of alcohol in our previous candidate gene approach,[11] we re-evaluated them via genome-wide exploration and newly examined the pleiotropic effects on metabolic traits (in the Amagasaki Study panel) using the refined (or extended) clinical/epidemiological data in the present study. Beside the 2 SNPs, 2 SNPs – rs10774610 and rs1725236 – showing significant ($P<5\times10^{-8}$) or suggestive ($P<5\times10^{-7}$) evidence of association in the GWA scan were taken forward to replication analysis in the Amagasaki Study (stage 2) panel. The genotype distribution of all tested SNPs was in Hardy–Weinberg equilibrium ($P>0.001$). We obtained successful genotyping call rates of >99% for the whole characterized sample.

Statistical Analysis

SNP Association Analysis A joint analysis strategy,[21] ie, a staged design in which part of the available samples are genotyped on a large number of SNP markers in stage 1, and part of these markers are later followed up by genotyping them on the remaining samples in stage 2, was adopted in the present study. The SNPs were tested for the association with alcohol drinking behavior (ever-drinkers vs. non-drinkers) in the GWA study panel (stage 1; 733 cases and 729 controls) and in the combined panel (stage 1+2; 4,780 cases and 1,940

Table 2. Effect Estimates of ALDH2 rs671 Genotype (AG vs. GG) Combined With the Status of Alcohol Drinking

rs671 genotype	Non-drinkers						Chance drinkers						Drinkers					
	GG (n=309)		AG (n=705)				GG (n=842)			AG (n=646)			GG (n=2,021)			AG (n=754)		
Tested trait	Estimate*	Estimate*	s.e.m.	P value	Estimate*	s.e.m.	P value	Estimate*	s.e.m.	P value	Estimate*	s.e.m.	P value	Estimate*	s.e.m.	P value	Estimate*	s.e.m.	P value
AST, IU/L	0	−0.38	0.44	0.377	−0.56	0.43	0.184	−0.93	0.45	0.034	1.40	0.41	8.5E-04	−0.42	0.45	0.346			
ALT, IU/L	0	0.16	0.61	0.784	−0.43	0.60	0.468	−1.09	0.63	0.071	1.13	0.57	0.050	−2.88	0.63	4.5E-07			
γGTP, IU/L	0	0.00	0.68	0.999	0.82	0.67	0.226	−0.70	0.71	0.298	9.19	0.63	4.5E-31	3.08	0.70	4.1E-05			
Triglycerides, mg/dl	0	3.63	3.24	0.264	−1.26	3.19	0.686	0.24	3.37	0.942	4.93	3.03	0.107	1.01	3.33	0.759			
LDL-C, mg/dl	0	1.52	2.17	0.486	−5.96	2.14	0.005	−1.62	2.26	0.473	−10.78	2.03	1.2E-07	−8.99	2.24	5.9E-05			
HDL-C, mg/dl	0	−1.36	1.11	0.213	1.18	1.10	0.284	−0.29	1.16	0.800	6.03	1.04	2.6E-08	4.38	1.14	1.9E-04			
Uric acid, mg/dl	0	−0.05	0.09	0.586	−0.01	0.09	0.922	−0.08	0.09	0.396	0.19	0.08	0.021	0.11	0.09	0.201			
FPG, mmol/L	0	−0.05	0.04	0.140	0.00	0.04	0.947	−0.04	0.04	0.296	0.03	0.03	0.310	−0.03	0.04	0.389			
SBP, mmHg	0	−1.37	1.02	0.180	−1.71	1.00	0.089	−3.41	1.05	0.001	1.04	0.95	0.277	−0.80	1.05	0.446			
DBP, mmHg	0	−0.33	0.65	0.609	−0.55	0.64	0.394	−1.30	0.67	0.053	2.10	0.61	5.9E-04	0.83	0.67	0.216			
BMI, kg/m²	0	−0.25	0.22	0.259	−0.12	0.22	0.597	−0.40	0.23	0.079	−0.36	0.21	0.080	−0.61	0.23	0.008			

Results for GG and AG genotype classes are shown for each category of drinking staus.
*Effects of rs671 genotype and alcohol drinking for the individual genotype classes are shown by the estimated changes with GG homozygote in the non-drinkers used as a reference group, after adjustment for sex, age and BMI.
Abbreviations see in Table 1.

Table 3. Estimated Effects of ALDH2 rs671 Genotype on Metabolic Traits by Regression Analysis With and Without Adjustment for Weekly Alcohol Intake

Tested trait	Predictor					
	ALDH2 rs671 [AG vs. GG] with adjustment for alcohol			ALDH2 rs671 [AG vs. GG] w/o adjustment for alcohol		
	β	SE	P value	β	SE	P value
AST, IU/L	−1.25	0.36	2.7E-04	−1.60	0.35	2.2E-06
ALT, IU/L	−3.14	0.48	5.5E-13	−3.20	0.47	6.4E-14
γGTP, IU/L	−2.92	0.57	1.0E-08	−3.81	0.58	3.1E-14
Triglycerides, mg/dl	0.45	2.58	0.861	−0.98	2.54	0.694
LDL-C, mg/dl	1.28	1.70	0.453	2.50	1.68	0.136
HDL-C, mg/dl	−2.03	0.87	0.017	−2.79	0.86	8.5E-04
Uric acid, mg/dl	−0.04	0.07	0.639	−0.06	0.07	0.410
FPG, mmol/L	−0.06	0.03	0.027	−0.07	0.03	0.016
SBP, mmHg	−0.62	0.78	0.428	−1.45	0.77	0.062
DBP, mmHg	−0.45	0.51	0.378	−0.97	0.50	0.054

Linear regression analysis was performed using drinkers (n=1,741) with sufficient information on weekly alcohol intake ('gou' per week) after adjustment for sex, age and BMI.
For the individual traits, subjects who took treatment for the corresponding disorder are not included in the analysis. As for triglycerides and FPG, subjects who did not keep fasting condition (>6 h) at the examination are not included (n=3 and n=8 for triglycerides and FPG, respectively).
Abbreviations see in Table 1.

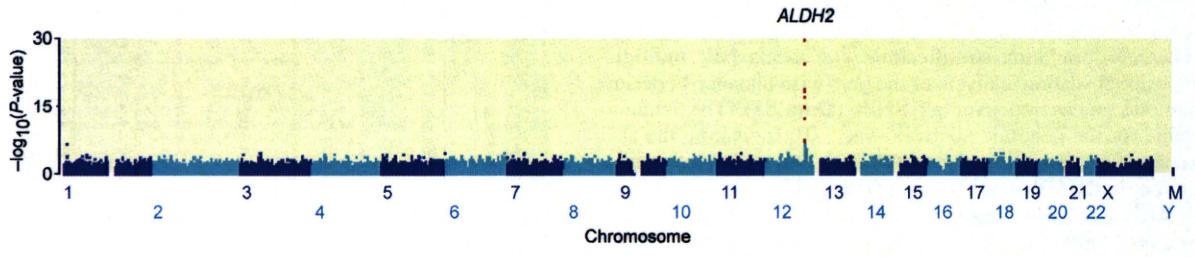

Figure 1. Manhattan plot of GWA study for alcohol drinking behavior.

controls) by logistic regression analysis after adjustment for sex, age (GWA study and Amagasaki Study panels) and enrolment site (GWA study panel only). To combine the association results from the 2 panels, we used the inverse variance method (Table S1).

Positive Selection in the 12q24 Region Near ALDH2

While the present GWA study identified strong associations at several SNP loci in a >0.8-Mb interval on 12q24, they became no more significant after adjustment for rs671. Since a previous European GWA study on hematological traits claimed significant evidence for signatures of natural selection in the corresponding region on 12q24,[22] we hypothesized that a long-range, evolutionarily derived haplotype, upon which effect alleles (A of rs671 with effect toward moderation of alcohol intake) could lie, arose from a positive selection in the Japanese. To test this hypothesis, we performed haplotype-based tests[23] in the 12q24 region near *ALDH2*.

Effect Estimates of rs671 Genotype on Metabolic Traits

We performed multiple regression analysis to test the effects of *ALDH2* rs671 genotype (AG vs. GG) on metabolic traits with adjustment for drinking status, sex and smoking habits as covariates (Table S2). Further, we evaluated the effects of *ALDH2* rs671 genotype on metabolic traits in 2 ways: by treating drinkers as a category (Table 2); and by quantitative adjustment for weekly alcohol intake among drinkers (Table 3). To adjust for skewness in the distribution, AST, ALT, γGTP, triglycerides and HDL-C were log-transformed before regression analysis; the effect size estimates were then re-transformed to the original units.

Results

Characteristics of participants in the present study are summarized in Table 1. In the general population (Amagasaki Study) panel, there were various differences among 3 groups classified by drinking behavior. Chance drinkers were significantly (P<0.05) younger than both drinkers and non-drinkers. Concentrations of serum liver enzymes (AST, ALT, and γGTP) and triglyceride were significantly higher in drinkers than in chance drinkers and non-drinkers. However, low-density lipoprotein cholesterol (LDL-C) levels were significantly lower in both drinkers and chance drinkers than in non-drinkers. Despite some differences in the ratio of people under treatment, concentrations of serum uric acid, plasma glucose, and blood pressure tended to be higher in drinkers as compared to those in non-drinkers. Apart from such inter-group differences in objective variables, smoking behavior largely differed among the 3 groups, party due to the increased ratio of men to women in drinkers.

In the genome-wide exploration (stage 1), there was a

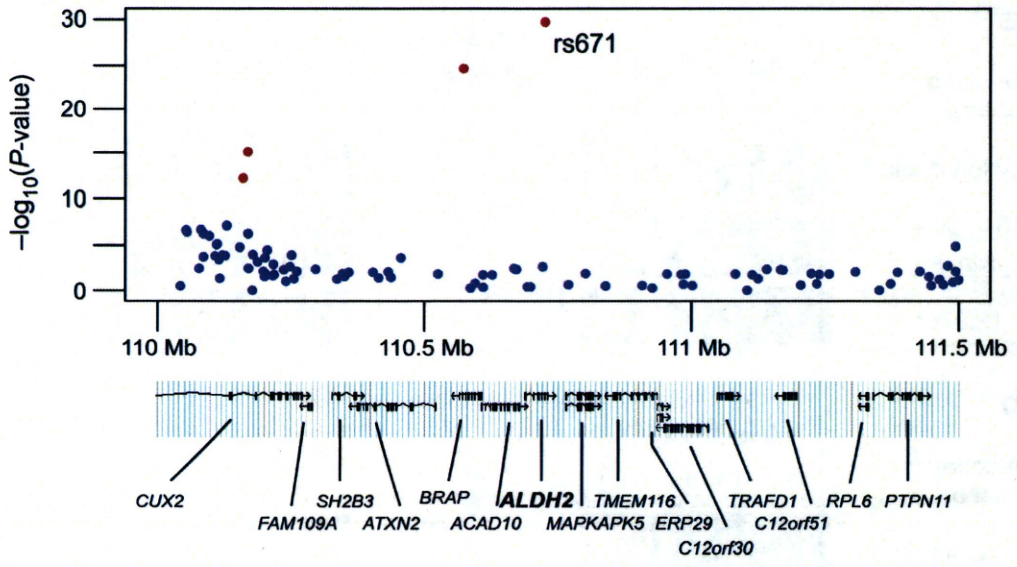

Figure 2. Plots of alcohol drinking behavior association in a 12q24 region near the *ALDH2* locus. All genotyped SNPs in the current Japanese GWA scan are plotted with their $-\log_{10}$ (P values) for alcohol drinking behavior against chromosome positions (in Mb). SNPs attaining genome-wide significance ($P<5\times10^{-8}$) are colored in red.

single prominent peak on 12q24 (Figure 1); the strongest evidence of association with drinking behavior was identified for rs671 in *ALDH2* (odds ratio (OR)=0.31, $P=1.8\times10^{-30}$) among significant association signals (a total 12 SNPs showing $P<5\times10^{-8}$), which all clustered in the 12q24 region (Figure 2 and Table S3). With the genotype data adjusted for rs671, most of the association signals became no more significant apart from 2 SNPs, rs10774610 and rs11065774 ($r^2=0.97$ between the SNPs), at which a borderline ($P<0.05$) level of association remained. In the joint analysis (stage 1+2), these associations were proven not to be independent of rs671 (Table S1). Besides, although a SNP, rs1725236, showed suggestive ($P<5\times10^{-7}$) association in stage-1 GWA scan, we could not verify it in the joint analysis. Instead, we could replicate a nominally significant association (OR=1.20, $P=3.6\times10^{-4}$ in the joint analysis) at rs1229984 in *ADH1B*, a candidate gene SNP previously reported (Table S1).[11,24,25]

On 12q24, rs671 was found to be in strong linkage disequilibrium with rs3782886 in *BRAP* ($r^2=0.87$). These 2 SNPs and 6 other SNPs (rs11066001, rs11066015, rs4646776, rs11066132, rs2074356, and rs11066280) are polymorphic only in East Asians and they constituted the evolutionarily derived haplotype from the HapMap data. A long-range haplotype (>0.7 Mb estimated from these 8 SNPs) was hypothesized to have arisen from a positive selection specific to East Asians; this was supported by the slower decreases in extended haplotype homozygosity[26] for the derived alleles than the ancestral alleles in the 12q24 region (Data S1 and Figure S2). Thereafter, we focused on rs671 because (1) it had the strongest association signal among the SNPs tested in stage-1 GWA scan, (2) the association of other individual SNPs did not remain significant when conditioning on rs671, and (3) there had been substantial evidence supporting the functional importance of rs671 (regarding the enzyme activity of ALDH2) in ethanol metabolism.

We then verified genetic impacts of rs671 on drinking behavior using Amagasaki Study samples in 2 analytical procedures, ie, categorical (or drinking status) and quantitative (in tertiles) classifications. According to drinking status–non-drinker, chance drinker, and drinker–genotype distribution of rs671 significantly differed in both sexes (eg, $P=2\times10^{-219}$ for men and $P=2\times10^{-72}$ for women, non-drinker vs. drinker) (Figure 3a). Those with AA homozygote of rs671 are non-drinkers (n=325) except for 5 drinkers and 22 chance drinkers, according to the definition of drinking status in the present study. When drinkers (2,206 men and 588 women) were further categorized into 3 tertiles according to the amount of alcohol intake, clear stepwise increases in the G (alcohol tolerant-type)-allele frequency were observed in men (75% to 90%; eg, $P=1\times10^{-15}$, 1st vs. 3rd tertile) and women (85% to 94%; eg, P=0.018, 1st vs. 3rd tertile) (Figure 3b). Also, such a correlation between allele/genotype of rs671 and the amount of alcohol intake could be detected when the drinkers were more quantitatively grouped into 5 strata (Figure S3).

In the context of gene–environment interaction, we investigated genetic effects of rs671 for a series of metabolic traits in 2 ways. First, when we treated drinkers as a single category, there were significant inter-genotype (GG vs. AG) differences in concentrations of serum liver enzymes–AST, ALT, and γGTP–by interacting with alcohol intake (Table S2). Of note is the fact that, as compared to GG-homozygous non-drinkers (Table 2 and Figure S4), ALT concentrations were significantly ($P<0.05$) increased among GG-homozygous drinkers and significantly ($P=4.5\times10^{-7}$) decreased among AG-heterozygous drinkers; diastolic blood pressure was significantly ($P=5.9\times10^{-4}$) increased among GG-homozygous drinkers but did not significantly change among AG-heterozygous drinkers. There were no significant inter-genotype (GG vs. AG) differences in BMI despite its potential interaction with alcohol intake (Table 2 and Table S2). Next, when we focused on drinkers to consider the effect of individual alcohol intake, inter-genotype (GG vs. AG) differences were still significant

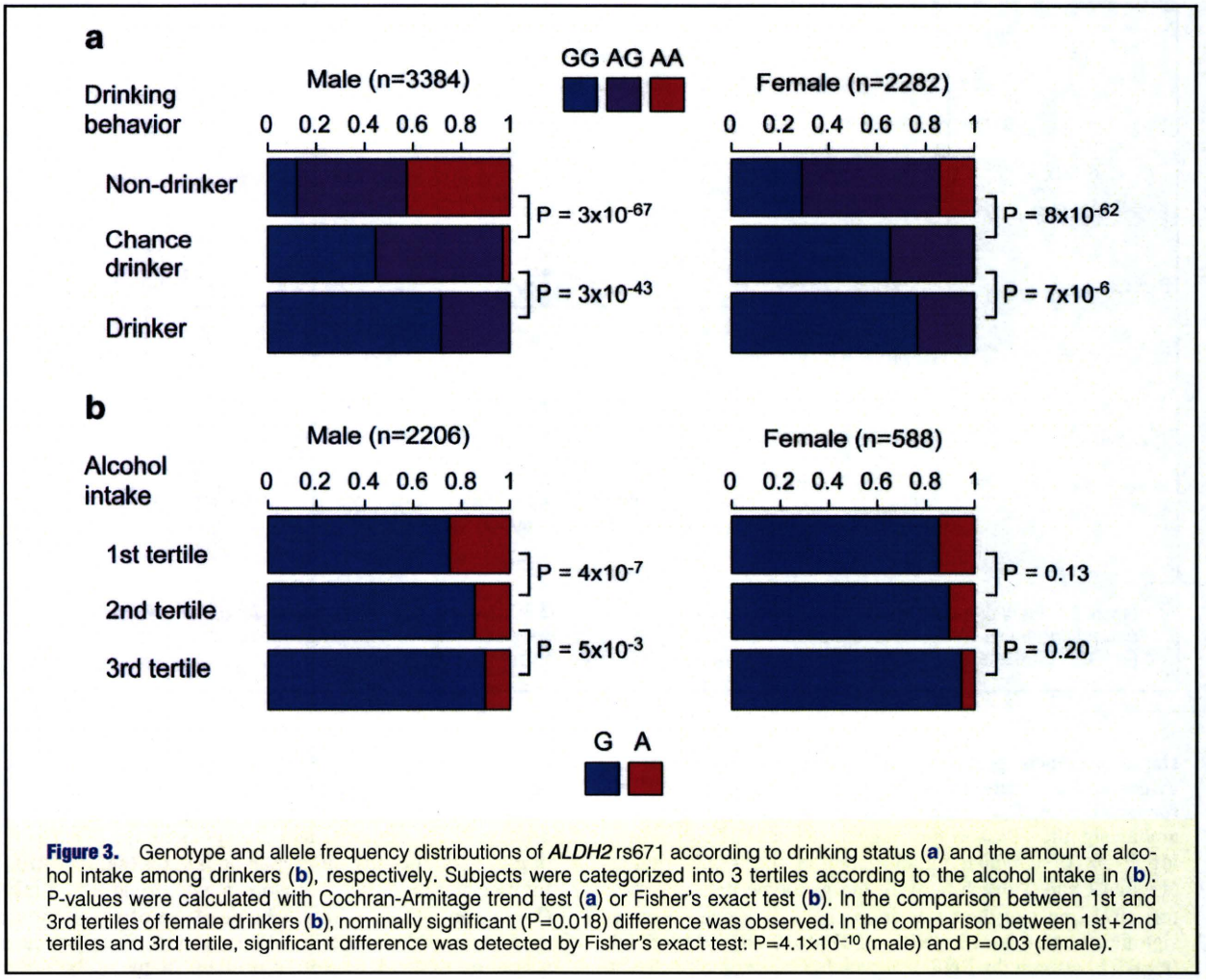

Figure 3. Genotype and allele frequency distributions of *ALDH2* rs671 according to drinking status (**a**) and the amount of alcohol intake among drinkers (**b**), respectively. Subjects were categorized into 3 tertiles according to the alcohol intake in (**b**). P-values were calculated with Cochran-Armitage trend test (**a**) or Fisher's exact test (**b**). In the comparison between 1st and 3rd tertiles of female drinkers (**b**), nominally significant (P=0.018) difference was observed. In the comparison between 1st+2nd tertiles and 3rd tertile, significant difference was detected by Fisher's exact test: $P=4.1\times10^{-10}$ (male) and P=0.03 (female).

($P=2.7\times10^{-4}$ to 5.5×10^{-13} after adjustment for alcohol intake) in concentrations of serum liver enzymes and also marginally significant ($P<0.05$) in levels of serum HDL-C and plasma glucose (**Table 3**).

Discussion

The present study has confirmed *ALDH2* as a major locus regulating drinking behavior in the Japanese population. This is the first GWA study published to date, which investigates genetic susceptibility to normative alcohol use. Although it did not attain statistical significance in the exploration stage, the association was also replicated for a candidate gene SNP, rs1229984 (in *ADH1B*), as previously reported.[11,24,25] This indicates the presence of susceptibility genes other than *ALDH2* with relatively modest effects on drinking behavior. Important findings in our study came from the systematic examination of the association of *ALDH2* with cardiovascular risk factors related to alcohol intake. Notably, our results have revealed that rs671 (in *ALDH2*) exerts substantial genetic impacts not only on drinking behavior but also on several alcohol-related metabolic traits including HDL-C and liver enzymes, at least in part, independently of the amount of alcohol intake among drinkers.

A number of previous studies have reported the relevance of *ALDH2* to the risk of developing alcoholism in East Asians.[27,28] Flushing and discomfort, such as headache and nausea, occur in many individuals of East Asian ancestry after drinking even small amounts of ethanol. This has been known to be related to a functional variant in the *ALDH2* gene (*ALDH2* 504Lys), a substitution of the Glu at codon position 504 with Lys,[29] which corresponds to the A-allele of rs671. This variant allele acts as a dominant negative with the heterozygote having considerably reduced enzyme activity and the homozygote having no activity. The major metabolic pathway for ethanol is degradation by ADH (alcohol dehydrogenase) enzymes to an intermediate metabolite, acetaldehyde, followed by its degradation to acetate by ALDH (aldehyde dehydrogenase) enzymes. There are 2 major ALDH isozymes in the liver: cytosolic ALDH1 and mitochondrial ALDH2. The reduced activity of ALDH2 can lead to increase in the transient concentrations of the toxic acetaldehyde after drinking, thereby protecting the individuals with the *ALDH2* 504Lys variant(s) from heavy drinking.

Besides the identification of *ALDH2* 504Lys as a major genetic determinant of drinking behavior in the Japanese via genome-wide exploration, the present study has provided insights into metabolic functions of *ALDH2* 504Lys among the variant carriers (principally AG heterozygote of rs671) of drinkers. In epidemiological studies, most of the apparent

benefit of moderate alcohol consumption on the risk of coronary artery disease (or myocardial infarction) has been attributed to an increase in HDL-C levels.[2,30,31] In accordance with the previous study by Nakamura et al,[32] HDL-C concentrations were significantly lower in the AG heterozygotes than the wild-type form homozygotes (GG) among drinkers (Table 3); this association was more prominent in heavier drinkers (β= –3.92 mg/dl, AG vs. GG in the upper tertile; Table S4) and in men (Table S5). Since female drinkers tend to have a moderate amount of alcohol consumption (Figure S1), the observed sexual dimorphism could be explained by a dose-dependent interaction between *ALDH2* rs671 and HDL-C concentrations. Another Japanese study[33] reported the association between *ALDH2* 504Lys and low HDL-C concentrations among non-drinkers; while there was the same direction of association (β=–1.36 mg/dl, AG vs. GG), the estimated effects of *ALDH2* rs671 genotype for HDL-C concentrations did not reach a nominal significance level in the present study (Table 2 and Table S6). In contrast to HDL-C levels, LDL-C levels were significantly decreased among drinkers as compared to non-drinkers in the general population panel, in accordance with the previous report in the Japanese.[32] That is, LDL-C levels were significantly higher in those with AG heterozygote than those with GG homozygote only among chance drinkers (β=4.01 mg/dl, P=0.012; Table S6), whereas this genotype–trait association was less prominent among drinkers (Tables 2,3). There were no significant differences in triglyceride concentrations between the rs671 genotype classes. Thus, despite the relatively modest strength of association, the *ALDH2* 504Lys variant confers overall genetic effects on lipid profile in the direction for atherogenesis; ie, a decrease in HDL-C levels and an increase in LDL-C levels. This may account for, at least in part, a potential biological link between ALDH2 and cardiac ischemia as reported by Chen et al,[34] who identified ALDH2 as an enzyme whose activation correlated with reduced ischemic heart damage in rodent models.

It has to be noted that, although statistical significance appeared to be borderline, blood pressure was lower in those with AG heterozygote than those with GG homozygote among chance drinkers (β=–1.70 mmHg for systolic blood pressure, AG vs. GG (P=0.018); Table S6), in accordance with our previous report.[11] Among non-drinkers, or drinkers stratified by the amount of alcohol intake, the genetic effect on blood pressure was not statistically significant (Tables S4, S6). Our data cannot provide sufficient evidence supporting the notion that this locus differently exerts pressor effects between heavy drinkers and non-heavy drinkers (data not shown). Moreover, we found that concentrations of serum liver enzymes (in particular, ALT) were significantly lower in those with AG heterozygote than those with GG homozygote among drinkers (Tables 2,3). This association remains highly significant among drinkers after adjustment for alcohol intake (Table 3); it is highly significant (P=3.2×10^{-6} to 4.3×10^{-14}) for all 3 parameters in men and significant (P=9.8×10^{-4}) only for γGTP in women (Table S5). The findings of sexual dimorphism could be related to differences in the average amount of alcohol consumption between men and women (Figure S1). These 'protective' effects on liver tissue (or fatty liver disease) are in good agreement with a previous study in Japanese male workers[35] and the study of *Aldh2* knockout mice.[36] Taken together, *ALDH2* 504Lys might exert some genetic effects on metabolic traits other than lipids in the direction against atherogenesis. Further investigation is warranted to clarify such pleiotropic or bi-directional functions of *ALDH2* 504Lys among drinkers.

Several studies including ours (Figure S2)[37,38] have supported the hypothesis that positive selection has operated on *ALDH2* 504Lys; however, conclusive results have not been obtained thus far, partly due to the current methodological limitations.[39] The restricted geographic distribution and high frequency of the physiologically important *ALDH2* 504Lys allele in East Asia has been of longstanding interest to human population geneticists. Additional information on genomic structure will be available from the ongoing 1,000 genomes project (http://www.1000genomes.org/; accessed on 9 Feb 2011) and helps to resolve this debated issue.

There are some limitations in the present study with regard to study design and data interpretation. We attempted to explore genetic factors influencing alcohol drinking behavior in normative alcohol use. Although we used the total units of ethanol for the weekly intake as a measure of alcohol intake in the quantitative analysis, this indicator of drinking behavior seems to be rather subjective, eg, the frequency of drinking on weekdays can be largely influenced by factors other than alcohol tolerance. In the general population, drinking behavior (or alcohol preference) is supposed to be defined by a combination of factors including genetic and environmental factors. That is, besides age and sex, a number of socio-economic factors such as occupation, place of residence, income, and lifestyle, the information of which is unavailable in the present study, exert some effects on the individual drinking behavior. Further, comprehensive multivariate analysis including the non-genetic factors as covariates is warranted to evaluate the relative contribution of alcohol intolerance, which is substantially defined by the *ALDH2* 504Lys allele, to the inter-individual variation in drinking behavior.

In summary, the present GWA study has identified the *ALDH2* 504Lys allele and haplotype as a major determinant of genetic susceptibility to normative alcohol use in the Japanese. People with *ALDH2* 504Lys homozygote cannot tolerate the aversive effects of alcohol in principle. Those with heterozygote genotype are also less likely to become drinkers; once they regularly drink some amounts of alcohol, bi-directional influences of the variant on atherogenesis will be brought about with relation to cardiovascular disease.

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References

1. Alkerwi A, Boutsen M, Vaillant M, Barre J, Lair ML, Albert A, et al. Alcohol consumption and the prevalence of metabolic syndrome: A meta-analysis of observational studies. *Atherosclerosis* 2009; **204:** 624–635.
2. Rimm EB, Williams P, Fosher K, Criqui M, Stampfer MJ. Moderate alcohol intake and lower risk of coronary heart disease: Meta-analysis of effects on lipids and haemostatic factors. *BMJ* 1999; **319:** 1523–1528.
3. Berger K, Ajani UA, Kase CS, Gaziano JM, Buring JE, Glynn RJ, et al. Light-to-moderate alcohol consumption and risk of stroke among U.S. male physicians. *N Engl J Med* 1999; **341:** 1557–1564.
4. Koppes LL, Dekker JM, Hendriks HF, Bouter LM, Heine RJ. Moderate alcohol consumption lowers the risk of type 2 diabetes: A meta-analysis of prospective observational studies. *Diabetes Care* 2005; **28:** 719–725.

5. Xin X, He J, Frontini MG, Ogden LG, Motsamai OI, Whelton PK. Effects of alcohol reduction on blood pressure: A meta-analysis of randomized controlled trials. *Hypertension* 2001; **38:** 1112–1117.
6. Kato I, Kiyohara Y, Kubo M, Tanizaki Y, Arima H, Iwamoto H, et al. Insulin-mediated effects of alcohol intake on serum lipid levels in a general population: The Hisayama Study. *J Clin Epidemiol* 2003; **56:** 196–204.
7. Rose RJ, Dick DM, Viken And RJ, Kaprio J. Gene-environment interaction in patterns of adolescent drinking: Regional residency moderates longitudinal influences on alcohol use. *Alcohol Clin Exp Res* 2001; **25:** 637–643.
8. Bierut LJ, Agrawal A, Bucholz KK, Doheny KF, Laurie C, Pugh E, et al. A genome-wide association study of alcohol dependence. *Proc Natl Acad Sci USA* 2010; **107:** 5082–5087.
9. Crabbe JC, Bell RL, Ehlers CL. Human and laboratory rodent low response to alcohol: Is better consilience possible? *Addict Biol* 2010; **15:** 125–144.
10. Froehlich JC. What aspects of human alcohol use disorders can be modeled using selectively bred rat lines? *Subst Use Misuse* 2010; **45:** 1727–1741.
11. Tsuchihashi-Makaya M, Serizawa M, Yanai K, Katsuya T, Takeuchi F, Fujioka A, et al. Gene-environmental interaction regarding alcohol-metabolizing enzymes in the Japanese general population. *Hypertens Res* 2009; **32:** 207–213.
12. Linneberg A, Gonzalez-Quintela A, Vidal C, Jørgensen T, Fenger M, Hansen T, et al. Genetic determinants of both ethanol and acetaldehyde metabolism influence alcohol hypersensitivity and drinking behaviour among Scandinavians. *Clin Exp Allergy* 2010; **40:** 123–130.
13. Chen YC, Peng GS, Wang MF, Tsao TP, Yin SJ. Polymorphism of ethanol-metabolism genes and alcoholism: Correlation of allelic variations with the pharmacokinetic and pharmacodynamic consequences. *Chem Biol Interact* 2009; **178:** 2–7.
14. Yamada Y, Imai T, Ishizaki M, Honda R. ALDH2 and CYP2E1 genotypes, urinary acetaldehyde excretion and the health consequences in moderate alcohol consumers. *J Hum Genet* 2006; **51:** 104–111.
15. Hiura Y, Tabara Y, Kokubo Y, Okamura T, Goto Y, Nonogi H, et al. Association of the functional variant in the 3-hydroxy-3-methylglutaryl-coenzyme a reductase gene with low-density lipoprotein-cholesterol in Japanese. *Circ J* 2010; **74:** 518–522.
16. Takeuchi F, Isono M, Katsuya T, Yamamoto K, Yokota M, Sugiyama T, et al. Blood pressure and hypertension are associated with 7 loci in the Japanese population. *Circulation* 2010; **121:** 2302–2309.
17. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, et al. PLINK: A tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 2007; **81:** 559–575.
18. Takeuchi F, Serizawa M, Yamamoto K, Fujisawa T, Nakashima E, Ohnaka K, et al. Confirmation of multiple risk Loci and genetic impacts by a genome-wide association study of type 2 diabetes in the Japanese population. *Diabetes* 2009; **58:** 1690–1699.
19. Devlin B, Roeder K. Genomic control for association studies. *Biometrics* 1999; **55:** 997–1004.
20. Takeuchi F, Kashida M, Okazaki O, Tanaka Y, Fukuda S, Kashima T, et al. Evaluation of pharmacogenetic algorithm for warfarin dose requirements in Japanese patients. *Circ J* 2010; **74:** 977–982.
21. Skol AD, Scott LJ, Abecasis GR, Boehnke M. Joint analysis is more efficient than replication-based analysis for two-stage genome-wide association studies. *Nat Genet* 2006; **38:** 209–213.
22. Soranzo N, Spector TD, Mangino M, Kühnel B, Rendon A, Teumer A, et al. A genome-wide meta-analysis identifies 22 loci associated with eight hematological parameters in the HaemGen consortium. *Nat Genet* 2009; **41:** 1182–1190.
23. Pickrell JK, Coop G, Novembre J, Kudaravalli S, Li JZ, Absher D, et al. Signals of recent positive selection in a worldwide sample of human populations. *Genome Res* 2009; **19:** 826–837.
24. Carr LG, Foroud T, Stewart T, Castelluccio P, Edenberg HJ, Li TK. Influence of ADH1B polymorphism on alcohol use and its subjective effects in a Jewish population. *Am J Med Genet* 2002; **112:** 138–143.
25. Macgregor S, Lind PA, Bucholz KK, Hansell NK, Madden PA, Richter MM, et al. Associations of ADH and ALDH2 gene variation with self report alcohol reactions, consumption and dependence: An integrated analysis. *Hum Mol Genet* 2009; **18:** 580–593.
26. Voight BF, Kudaravalli S, Wen X, Pritchard JK. A map of recent positive selection in the human genome. *PLoS Biol* 2006; **4:** e72.
27. Goedde HW, Agarwal DP, Harada S, Meier-Tackmann D, Ruofu D, Bienzle U, et al. Population genetic studies on aldehyde dehydrogenase isozyme deficiency and alcohol sensitivity. *Am J Hum Genet* 1983; **35:** 769–772.
28. Crabb DW. Biological markers for increased risk of alcoholism and for quantitation of alcohol consumption. *J Clin Invest* 1990; **85:** 311–315.
29. Harada S, Misawa S, Agarwal DP, Goedde HW. Liver alcohol dehydrogenase and aldehyde dehydrogenase in the Japanese: Isozyme variation and its possible role in alcohol intoxication. *Am J Hum Genet* 1980; **32:** 8–15.
30. Hulley SB, Gordon S. Alcohol and high-density lipoprotein cholesterol: Causal inference from diverse study designs. *Circulation* 1981; **64:** III57–III63.
31. Gaziano JM, Buring JE, Breslow JL, Goldhaber SZ, Rosner B, VanDenburgh M, et al. Moderate alcohol intake, increased levels of high-density lipoprotein and its subfractions, and decreased risk of myocardial infarction. *N Engl J Med* 1993; **329:** 1829–1834.
32. Nakamura Y, Amamoto K, Tamaki S, Okamura T, Tsujita Y, Ueno Y, et al. Genetic variation in aldehyde dehydrogenase 2 and the effect of alcohol consumption on cholesterol levels. *Atherosclerosis* 2002; **164:** 171–177.
33. Wada M, Daimon M, Emi M, Iijima H, Sato H, Koyano S, et al. Genetic association between aldehyde dehydrogenase 2 (ALDH2) variation and high-density lipoprotein cholesterol (HDL-C) among non-drinkers in two large population samples in Japan. *J Atheroscler Thromb* 2008; **15:** 179–184.
34. Chen CH, Budas GR, Churchill EN, Disatnik MH, Hurley TD, Mochly-Rosen D. Activation of aldehyde dehydrogenase-2 reduces ischemic damage to the heart. *Science* 2008; **321:** 1493–1495.
35. Takeshita T, Yang X, Morimoto K. The ALDH2 genotype, alcohol intake, and liver-function biomarkers among Japanese male workers. *Hum Genet* 2000; **106:** 589–593.
36. Matsumoto A, Kawamoto T, Mutoh F, Isse T, Oyama T, Kitagawa K, et al. Effects of 5-week ethanol feeding on the liver of aldehyde dehydrogenase 2 knockout mice. *Pharmacogenet Genomics* 2008; **18:** 847–852.
37. Oota H, Pakstis AJ, Bonne-Tamir B, Goldman D, Grigorenko E, Kajuna SL, et al. The evolution and population genetics of the ALDH2 locus: Random genetic drift, selection, and low levels of recombination. *Ann Hum Genet* 2004; **68:** 93–109.
38. Brown BT, Woerner A, Wilder JA. Ascertainment bias and the pattern of nucleotide diversity at the human ALDH2 locus in a Japanese population. *J Mol Evol* 2007; **64:** 375–385.
39. Li H, Borinskaya S, Yoshimura K, Kal'ina N, Marusin A, Stepanov VA, et al. Refined geographic distribution of the oriental ALDH2* 504Lys (nee 487Lys) variant. *Ann Hum Genet* 2009; **73:** 335–345.

Supplemental Files

Supplemental File 1

Data S1. Supplemental Note

Table S1. Loci Identified or Tested for Association With Drinking Behavior in the Japanese Population

Table S2. Estimated Effects of ALDH2 rs671 Genotype on Metabolic Traits by Regression Analysis With Adjustment for Drinking Status and Sex as Covariates

Table S3. Association With Drinking Behavior in the 12q24 Region

Table S4. Estimated Effects of ALDH2 rs671 Genotype on Metabolic Traits by Regression Analysis in Drinkers Stratified by Tertiles of Weekly Alcohol Intake

Table S5. Estimated Effects of ALDH2 rs671 Genotype on Metabolic Traits by Regression Analysis With and Without Adjustment for Weekly Alcohol Intake After Stratification by Sex

Table S6. Estimated Effects of ALDH2 rs671 Genotype on Metabolic Traits Among Chance Drinkers and Non-Drinkers

Figure S1. Histograms of weekly alcohol intake among drinkers: total sample (n=1,741) in the top left, men (n=1,311) in the top right, and women (n=430) in the bottom.

Figure S2. Suggestive evidence for positive selection in a 12q24 region near the *ALDH2* locus.

Figure S3. Allele frequency (**a**) and genotype (**b**) distributions of *ALDH2* rs671 according to the amount of alcohol intake among drinkers.

Figure S4. Estimated changes in metabolic traits according to rs671 genotype (GG vs. AG) and drinking status.

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Blood Pressure and Hypertension Are Associated With 7 Loci in the Japanese Population

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Background—Two consortium-based genome-wide association studies have recently identified robust and significant associations of common variants with systolic and diastolic blood pressures in populations of European descent, warranting further investigation in populations of non-European descent.

Methods and Results—We examined the associations at 27 loci reported by the genome-wide association studies on Europeans in a screening panel of Japanese subjects (n=1526) and chose 11 loci showing association signals (1-tailed test in the screening, $P<0.3$) for an extensive replication study with a follow-up panel of 3 Japanese general-population cohorts (n ≤24 300). Significant associations were replicated for 7 loci—*CASZ1*, *MTHFR*, *ITGA9*, *FGF5*, *CYP17A1-CNNM2*, *ATP2B1*, and *CSK-ULK3*—with any or all of these 3 traits: systolic blood pressure ($P=1.4\times10^{-14}$ to 0.05), diastolic blood pressure ($P=1.9\times10^{-12}$ to 0.05), and hypertension ($P=2.0\times10^{-14}$ to 0.006; odds ratio, 1.10 to 1.29). The strongest association was observed for *FGF5*. In the whole study panel, the variance (R^2) for blood pressure explained by the 7 single-nucleotide polymorphism loci was calculated to be $R^2=0.003$ for male and 0.006 for female participants. Stratified analysis implied the potential presence of a gene-age-sex interaction, although it did not reach a conclusive level of statistical significance after adjustment for multiple testing.

Conclusions—We have confirmed 7 loci associated with blood pressure and/or hypertension in the Japanese. These loci can guide fine-mapping efforts to pinpoint causal variants and causal genes with the integration of multiethnic results. (***Circulation.*** **2010;121:2302-2309.)**

Key Words: blood pressure ■ genes ■ genetics ■ hypertension

Hypertension affects one third of the adult population worldwide and is a major risk factor of cardiovascular disease.[1] Even within the normal range, increases in blood pressure are associated with the risk of death from vascular diseases such as stroke and ischemic heart disease.[2] Although lifestyle influences (eg, excess salt and alcohol intake and lack of exercise) are known to increase blood pressure and the risk of developing hypertension, a substantial contribution of genetic factors to the overall disease pathogenesis has been documented by a number of epidemiological studies.[3,4] Despite considerable efforts to study the molecular genetics of hypertension, the inherently complex nature has hampered progress in elucidating the involved genes.

Clinical Perspective on p 2309

The recent advent of genome-wide association (GWA) studies has enabled identification of common variants associated with common diseases and traits.[5] Several GWA studies have thus far found loci associated with hypertension or blood pressure, few of which have attained genome-wide significance levels (eg, $P<5\times10^{-8}$).[6] Therefore, blood pressure variation in the general population is assumably due to multiple variants with

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Table 1. Clinical Characteristics of the Study Sample

Study	n	Women, %	Age (SD), y	SBP (SD), mm Hg	DBP (SD), mm Hg	BMI (SD), kg/m^2	HTN, %	Antihypertensive Therapy, %
Stage 1: screening in GWA study sample								
Total subjects examined	1526	43.1	66.1 (8.0)	134.1 (20.3)	76.8 (12.0)	23.5 (3.3)	52.7	38.2
Cases for screening	403	37.0	63.3 (7.5)	149.0 (20.3)	85.3 (13.0)	24.8 (3.5)	100	74.7
Controls for screening	452	51.0	64.4 (7.1)	115.4 (10.5)	69.0 (8.2)	22.2 (2.7)	0	0
Stage 2: follow-up								
2a: replication study								
Population cohort								
Amagasaki Study*	5745	40.2	48.8 (12.6)	124.3 (18.1)	75.9 (11.6)	23.0 (3.2)	23.4	9.7
Case-control panel†								
Cases for follow-up	1750	40.0	58.9 (11.2)	157.1 (19.6)	96.3 (11.3)	24.5 (3.4)	100	73.5
Controls for follow-up	1776	44.1	60.8 (8.6)	117.9 (12.1)	73.5 (8.6)	22.4 (2.8)	0	0
2b: replication study								
Population cohort								
Fukuoka Cohort Study	12 569	54.9	62.6 (6.8)	138.9 (21.2)	83.9 (11.7)	23.1 (3.0)	57.9	23.9
KING Study	3975	56.9	63.6 (6.6)	132.3 (19.8)	76.9 (11.2)	22.9 (3.0)	48.4	24.3
Case-control panel								
Cases for follow-up	1544	49.9	56.2 (2.7)	153.2 (20.6)	94.7 (12.6)	24.1 (3.3)	100	56.9
Controls for follow-up	5055	67.4	60.8 (6.4)	115.7 (9.2)	72.0 (7.4)	21.9 (2.7)	0	0

HTN indicates hypertension.

*Part of the Amagasaki Study samples (n=414) are included in the GWA study panel.

†In the stage-2a case-control study panel, 204 cases and 897 controls were derived from the Amagasaki Study panel; the remaining subjects were enrolled independently.

small effects; very large study samples are needed to provide definitive evidence of the principal hypertension-susceptibility gene(s).

Furthermore, meta-analysis of multiple GWA studies can facilitate detection of the variants with modest effects. With regard to hypertension, 2 consortia, the Cohorts for Heart and Aging Research in Genome Epidemiology Consortium[6] and the Global Blood Pressure Genetics consortium,[7] have been formed to conduct meta-analyses of GWA studies in populations of ≈30 000 individuals of European descent in each, with follow-up analysis using genotypes from large cohorts (>80 000 samples when combined). These large-scale analyses have identified 13 loci showing genome-wide significant association with systolic (SBP) and diastolic (DBP) blood pressures and/or hypertension, together with a number of other loci showing suggestive association.

Given the appreciable ethnic differences in the clinical presentation of hypertension between populations of European and non-European ancestry,[8] it is essential to test the genetic associations previously identified for Europeans in the other populations. We therefore conducted a cross-population comparison of susceptibility loci in a Japanese population. We also examined the influences of sex, age, and obesity on the strength of genetic association with blood pressure and/or hypertension to validate a complex interplay of genes and these nongenetic factors as previously reported in Europeans.[4,9,10]

Methods

Study Populations

Detailed characteristics of the subjects analyzed in each stage of the study are described in the online-only Data Supplement and Table 1. Our genetic studies for blood pressure and hypertension were originally organized as part of an ongoing GWA study for cardiometabolic disorders among Japanese subjects; the multistage design is summarized in Figure 1. In stage 1 of the GWA scan, 1526 Japanese samples were genotyped with 456 825 single-nucleotide polymorphism (SNP) markers. From the loci reported for Europeans,[6,7] those showing a tendency for association ($P<0.3$ by 1-tailed test in stage 1) were chosen for genotyping in a replication (stage 2a) panel involving 5745 Japanese subjects (hereafter called the Amagasaki cohort) consecutively enrolled in a population-based setting as described elsewhere[11] and 2425 subjects primarily recruited for the case-control study (1750 cases and 1776 controls) in stage 2a. Subsequently, the loci showing a significant association ($P<0.05$ in stage 2a) were tested in another replication (stage 2b) panel comprising 2 cohorts of 12 569 and 3975 subjects (hereafter called the Fukuoka cohort and the Kita-Nagoya Genomic Epidemiology [KING] Study cohort, respectively) randomly selected from the Japanese general population.[12] From the stage 2b panel, 1544 cases and 5055 controls were further chosen for case-control comparison. All participants from these studies provided written informed consent, and the local ethics committees approved the protocols.

Blood pressure levels were classified according to the Japanese Society of Hypertension Guidelines for the Management of Hypertension (JSH2009) as described elsewhere.[13] Cases were enrolled from clinical practices or annual medical checkups at medical institutions and university hospitals in accordance with the uniformly defined criteria. These criteria included (1) SBP ≥160 mm Hg and/or DBP ≥100 mm Hg for untreated subjects (grade II and III hypertension in JSH2009); (2) receiving long-term antihypertensive treatments; (3) no secondary form of hypertension as evaluated by an extensive workup that included serum creatinine and electrolytes, chest radiography, ECG, urinalysis, and other hematologic screening tests; and (4) age of onset ≤60 years. Normotensive controls were defined as follows: SBP <130 mm Hg and DBP <85 mm Hg without antihypertensive treatments (normal blood pressure in JSH2009) and age ≥50 years.

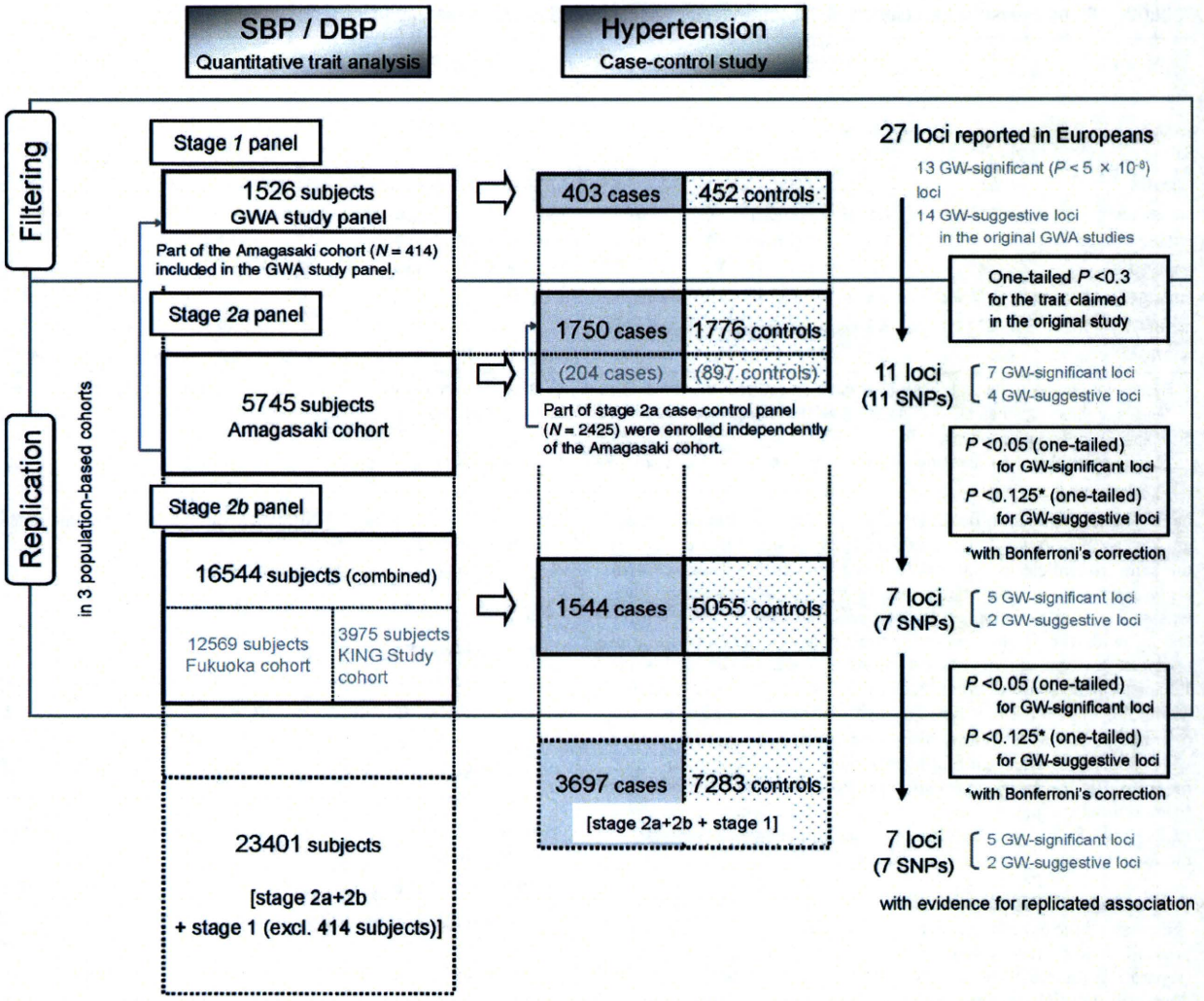

Figure 1. Flowchart summarizing the multistage design of the present study. Part of the Amagasaki Study samples (n=414) are included in the GWA study panel. In the stage 2a case-control study panel, 204 cases and 897 controls were derived from the Amagasaki Study panel, whereas the remaining subjects were independently enrolled. In stages 1 and 2b, the case-control study panel was derived from the quantitative trait analysis panel of the same stage. Thus, a total of 25 826 unrelated Japanese subjects (23 401 samples from the quantitative trait analysis panels in all stages plus 2425 samples independently enrolled in the stage 2a case-control study) were used for the entire study. In transitions from stage 2a to stage 2b and from stage 2b to final analysis (involving all stage panels), different cutoff levels were set for genome-wide (GW) –significant and GW-suggestive loci reported in the original GWA studies of Europeans.[6,7]

SNP Genotyping and Quality Control

In the stage 1 GWA scan, genotyping was performed with the Infinium HumanHap550 BeadArray (Illumina, San Diego, Calif), interrogating 555 352 SNPs (online-only Data Supplement). Data cleaning and analysis were performed with PLINK as described elsewhere.[14] Population stratification was checked by multidimensional scaling analysis of the pairwise distance between samples measured over all SNPs (online-only Data Supplement). The λ value for the genomic control was 1.00 to 1.02, indicating the absence of systematic confounding such as population stratification in the GWA study samples (see text and Figure I in the online-only Data Supplement).

In the replication study, samples were genotyped with the TaqMan assay for 11 SNPs from 11 unique loci previously identified in Europeans.[6,7] These included *CASZ1* (rs880315), *MTHFR* (rs17367504), *ITGA9* (rs155524), *MDS1* (rs448378), *FGF5* (rs16998073), *EFCAB1* (rs1910252), *CACNB2* (rs11014166), *CYP17A1-CNNM2* (rs12413409), *PLEKHA7* (rs406890), *ATP2B1* (rs2681472), and *CSK-ULK3* (rs1378942). The genotypic distribution of all tested SNPs was in Hardy-Weinberg equilibrium ($P>10^{-3}$). We obtained successful genotyping call rates of >99% for the whole characterized sample.

Statistical Analyses

SNP Association Analysis

The SNPs were tested for associations with blood pressure and hypertension with linear regression analysis and the Cochran-Armitage trend test, respectively. In the linear regression models, we adjusted continuous SBP and DBP for age, age^2, and body mass index (BMI) separately by sex (all panels) and the sample enrollment site (GWA study panel only). For the individuals receiving antihypertensive therapies, blood pressure was imputed by adding 15 and 10 mm Hg for SBP and DBP, respectively.[7,15] For the replication study of candidate SNP loci, we set in the stage 1 screening a significance level of $P<0.3$ (1-tailed) for the trait(s) that could be claimed by the original GWA studies at the individual loci based on the power calculation (Figure II and Table I in the online-only Data Supplement). In stages 2a and 2b, 1-tailed values of $P<0.05$ were considered statistically significant for the loci previously shown to

Table 2. Blood Pressure Association at 7 Loci Showing Association Signals in the Japanese

SNP	Chr	Nearby Gene(s)	Risk/Nonrisk Allele*	Japanese SBP β (95% CI), mm Hg	Japanese SBP P	Japanese DBP β (95% CI), mm Hg	Japanese DBP P	European Descent, β (95% CI), mm Hg SBP	European Descent, β (95% CI), mm Hg DBP	Heterogeneity, P† SBP	Heterogeneity, P† DBP
rs880315	1	*CASZ1*	C/T	1.08 (0.70–1.46)	2.2×10^{-8}	0.79 (0.56–1.01)	4.9×10^{-12}	0.53 (0.29–0.77)	0.30 (0.10–0.50)	0.14	0.007
rs17367504	1	*MTHFR*	A/G	0.65 (0.07–1.24)	0.03	0.34 (−0.01–0.69)	0.05	0.85 (0.63–1.07)	0.50 (0.26–0.74)	0.15	0.28
rs155524	3	*ITGA9*	G/A	0.48 (0.00–0.97)	0.05	0.26 (−0.03–0.55)	0.07	0.56 (0.25–0.87)	0.32 (0.14–0.50)	0.41	0.53
rs16998073	4	*FGF5*	T/A	1.51 (1.12–1.89)	3.1×10^{-14}	0.82 (0.59–1.05)	1.9×10^{-12}	0.65 (0.43–0.87)	0.50 (0.40–0.60)	0.01	0.07
rs12413409	10	*CYP17A1-CNNM2*	G/A	1.58 (1.18–1.98)	1.4×10^{-14}	0.76 (0.53–1.00)	2.9×10^{-10}	1.16 (0.92–1.40)	0.56 (0.27–0.85)	0.93	0.61
rs2681472	12	*ATP2B1*	A/G	0.99 (0.62–1.35)	1.5×10^{-7}	0.43 (0.21–0.64)	1.2×10^{-4}	0.85 (0.60–1.10)	0.50 (0.34–0.66)	0.55	0.28
rs1378942	15	*CSK-ULK3*	C/A	0.44 (−0.01–0.89)	0.05	0.35 (0.09–0.62)	0.009	0.48 (0.30–0.66)	0.43 (0.35–0.51)	0.44	0.30

Results for European-descent populations were drawn from the studies by Levy et al[6] and Newton-Cheh et al.[7] Two-tailed *P* values are shown.

*Alleles are nominated as those in dbSNP Build 130 mapped on the positive strand of Human Genome Build 36.3. Designation of risk allele is the same as that previously reported in Europeans.

†The Cochran Q test for heterogeneity of per-allele effect on blood pressure standardized in each population.

have genome-wide significant ($P<5\times10^{-8}$) association in Europeans; for an association to be considered significant, it had to involve the same risk allele as that reported in Europeans and was accordingly assessed with the 1-tailed test. Otherwise, a significance level was set at 1-tailed value of $P<0.05$ after adjustment for multiple testing with the Bonferroni correction. For the quantitative trait analysis, the genetic effects estimated in each of the multistage Japanese panels were combined by using the inverse variance method, except for a few types of analyses mostly of gene-age-sex interaction, for which the subject data from 3 population cohorts were combined after calibrating intercohort differences in blood pressure. For the case-control study, cases and controls were pooled from multistage panels (online-only Data Supplement). We used PLINK, the R software, and the rmeta and meta packages to test for the associations.

Assessment of the Cumulative Effect of Risk Variants

We assessed the cumulative effect of multiple SNPs by using a blood pressure risk score,[6] which was a weighted sum across the SNPs (separately for SBP and DBP) combining the β coefficients and doses of the risk alleles. To illustrate a trend by grouping the individuals, the blood pressure risk score was rounded to 1 mm Hg for SBP (groups ≤−3 to ≥3) and 0.5 mm Hg for DBP (groups ≤−1.5 to ≥1.5), with each risk score representing deviations from the study mean. Within a risk score group, we calculated the mean and 95% confidence interval (CI) of empirical blood pressure.

The multilocus risk of hypertension for an individual was assessed as the sum of doses of the risk alleles weighted by the logarithm of the odds ratio (OR) at the SNPs. We simulated a population with 20% prevalence by using bootstrap sampling. The prevalence was drawn from the National Health and Nutrition Survey, Japan 2006 (survey data are available at http://www-bm.mhlw.go.jp/houdou/2008/04/dL/h0430–2c.pdf [in Japanese]). In this simulated population, we arranged the individuals in order of their multilocus risk, sorted them into 20 equal-sized groups (5% in each), and calculated the actual proportion of hypertensive subjects in each group. The mean and 95% CI of the group-wise prevalence were estimated on the basis of 1000 bootstrap sampling trials.

Test of Ethnic Diversity and Interaction With Sex, Age, and BMI

The per-allele effect size, β, of an SNP on blood pressure was compared between the ethnic groups and among the subgroups stratified by sex, age, and BMI. Blood pressure was standardized as a *z* score within each ethnic group before cross-population comparison. The interaction of effect estimates with ethnicity (Japanese versus European) and with sex, age, and BMI was analyzed by the Cochran Q test and ANOVA, respectively (online-only Data Supplement). To collectively assess the proportion of variance for blood pressure explained by an SNP, risk score, or BMI, we calculated the coefficient of determination (R^2).

Results

Screening of GWA Data

We found no significant association by genome-wide exploration in the Japanese (Figure III in the online-only Data Supplement). Using the stage 1 GWA data set, we then examined association signals at 27 unique loci for which significant or suggestive evidence of association was previously reported by 2 GWA meta-analyses of Europeans.[6,7] Although no individual locus had strong statistical significance, the SNP markers from 11 (of 27) loci showed a tendency for association (1-tailed test, $P<0.3$) in the direction concordant with that previously reported for Europeans (Tables II and III in the online-only Data Supplement).

Replication of Selected SNPs in the Japanese

In the follow-up study (stages 2a and 2b), we tested blood pressure and hypertension associations with 11 SNP loci selected via the stage 1 screening. First, in the Amagasaki cohort panel (n=5745), we found significant ($P<0.05$) associations of 5 SNPs with DBP and/or SBP (Table IV in the online-only Data Supplement). In the stage 2a case-control panel, significant associations with hypertension were detected for 6 SNPs: 4 of the SNPs replicated for the blood pressure association plus rs155524 (*ITGA9*) and rs1378942 (*CSK-ULK3*). These were further genotyped in the Fukuoka Cohort Study panel (n=12 569) and the KING Study panel (n=3975), and the genetic associations were confirmed for 7 SNP loci ($P<0.05$): *CASZ1* (rs880315), *MTHFR* (rs17367504), *ITGA9* (rs155524), *FGF5* (rs16998073), *CYP17A1-CNNM2* (rs12413409), *ATP2B1* (rs2681472), and *CSK-ULK3* (rs1378942) (Tables 2 and 3 and Table IV in the online-only Data Supplement). Here, 1-tailed $P<0.0125$ (0.05/4) was considered significant for 4 (of 11) typed SNP loci, *CASZ1, ITGA9, MDS1*, and *EFCAB1*, which showed suggestive association in Europeans. Despite modest intercohort fluctuations in the strength of association, the genetic associations were appreciably reproducible across all stages

Table 3. Hypertension Association at 7 Loci Showing Association Signals in the Japanese

					Japanese						
					RAF				European Descent		
SNP ID	Chr	Position	Nearby Gene(s)	Risk/Nonrisk Allele*	Cases	Controls	OR (95% CI)	*P*	RAF	OR (95% CI)	Heterogeneity, *P*†
rs880315	1	10 719 453	*CASZ1*	C/T	0.696	0.660	1.18 (1.11–1.26)	3.0×10^{-7}	0.36	1.09 (1.05–1.14)	0.04
rs17367504	1	11 785 365	*MTHFR*	A/G	0.897	0.895	1.05 (0.95–1.16)	0.32	0.84	1.12 (1.08–1.16)	0.23
rs155524	3	37 537 145	*ITGA9*	G/A	0.173	0.156	1.13 (1.04–1.22)	0.005	0.38	1.08 (1.04–1.13)	0.41
rs16998073	4	81 541 520	*FGF5*	T/A	0.333	0.287	1.29 (1.21–1.37)	2.0×10^{-14}	0.21	1.10 (1.07–1.13)	1.1×10^{-5}
rs12413409	10	104 709 086	*CYP17A1-CNNM2*	G/A	0.758	0.724	1.22 (1.14–1.31)	7.7×10^{-9}	0.91	1.16 (1.11–1.21)	0.20
rs2681472	12	88 533 090	*ATP2B1*	A/G	0.640	0.617	1.10 (1.04–1.17)	0.002	0.83	1.16 (1.12–1.21)	0.17
rs1378942	15	72 864 420	*CSK-ULK3*	C/A	0.810	0.795	1.11 (1.03–1.20)	0.006	0.36	1.10 (1.08–1.13)	0.79

RAF indicates risk allele frequency. SNP association was adjusted for BMI and sex with logistic regression. Two-tailed *P* values are shown. Results for European-descent populations were drawn from the studies by Levy et al[6] and Newton-Cheh et al.[7]

*Alleles are nominated as those in dbSNP Build 130 mapped on the positive strand of Human Genome Build 36.3. Designation of risk allele is the same as that previously reported in Europeans.

†The Woolf test of heterogeneity for OR in Japanese and European-decent populations.

and different population cohorts in the Japanese (Table IV and Figure IV in the online-only Data Supplement).

Ethnic Heterogeneity in Effect Sizes

Of 27 candidate loci, the direction of association was concordant at 37% (10 of 27) and inverted at 19% (5 of 27) across the 3 traits between 2 ethnic groups (Table II in the online-only Data Supplement). Moreover, of 13 loci that attained genome-wide significance levels in Europeans, association was robustly replicated at 38% (5 of 13), was not reproducible at 54% (7 of 13) with an average power of >0.5, and was not assessable at 8% (1 of 13; no polymorphism detected for SNPs at *SH2B3*) in the Japanese. Thus, the real rate of positive (true positive plus false negative) seems to be between 5 of 13 and 9 of 13 for the reported genome-wide significant loci.

Among the 7 loci replicated in the whole study panel, there was significant ($P<0.05$) interethnic heterogeneity for rs880315 (*CASZ1*) and rs16998073 (*FGF5*). The effect sizes for these loci were significantly larger in the Japanese than in Europeans (Tables 2 and 3 and Figure VA and VB in the online-only Data Supplement).

Cumulative Effect of 7 Associated Loci

Despite the small value of explained variance (R^2) at each risk locus, knowledge about multiple-risk loci would allow identification of individuals with accumulated genetic risk.[14] Therefore, we calculated empirical blood pressure levels with the study subjects categorized according to their blood pressure risk scores. Across the risk score groups, the mean level of SBP and DBP increased significantly in a stepwise fashion in the Japanese (Figure 2A; $P<10^{-33}$ for each trend). We also investigated the combined risk of hypertension on the basis of 7 associated loci by analysis of the resampled data set. We thus found a 1.9-fold variation in hypertension prevalence from the lowest to the highest estimated risk groups for the combination of 7 associated loci in our study (Figure 2B).

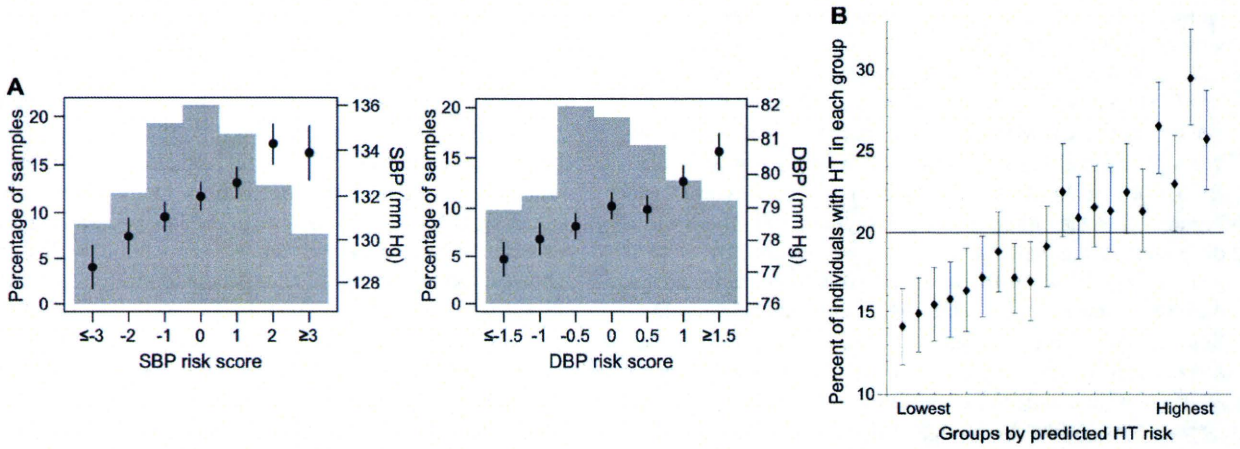

Figure 2. Estimation of the SBP and DBP risk scores (A) and the increase in hypertension (HT) risk (B) from the combination of the 7 variants replicated in this study. A, The mean (solid circles) and 95% CI (whiskers) were plotted. The sample sizes for each risk score group are shown by the shaded bars. The *P* values for the associations of the risk scores were highly significant: 2.9×10^{-37} (SBP vs SBP risk score), 6.0×10^{-34} (DBP vs DBP risk score). B, We used cases and controls with complete data from all stages of our study (n=10 811) and simulated a population with 20% prevalence. In this population, we arranged the individuals in order of their multilocus risk, sorted them into 20 equal-sized groups, and calculated the actual prevalence in each group. The mean and 95% CI of the group-wise prevalence were then plotted.

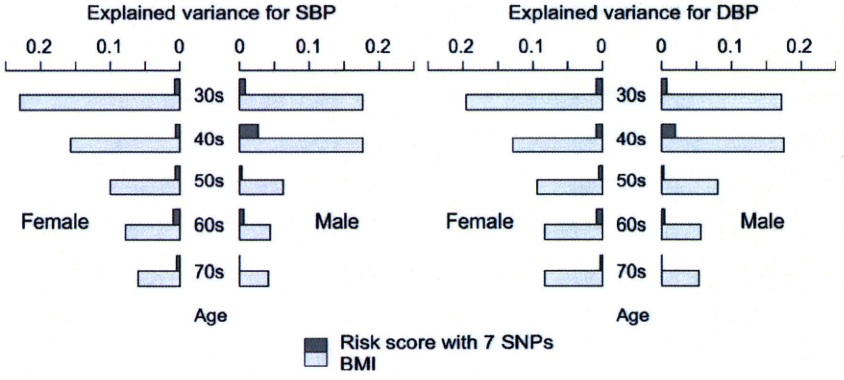

Figure 3. The variance (R^2) for SBP (left) and DBP (right) explained by BMI (light gray) and by genetic susceptibility (dark gray) represented by the risk score with the 7 SNPs. R^2 was calculated within each age class separately by sex.

Factors Modulating Genetic Associations

To estimate the impacts of risk factor variables on blood pressure variance in the Japanese, we first performed linear regression analysis with the stage 2a (Amagasaki Study) panel (Table V in the online-only Data Supplement). Sex was found to be a principal predictor of blood pressure ($P<10^{-36}$); hence, the subjects were analyzed separately by sex. In both sexes, age and age^2 were ranked in the first place (R^2=0.108 to 0.162) and BMI was in the second place (R^2=0.095 to 0.118) consistently for SBP and DBP. When the subjects were categorized into 5 age classes (30 to 39, 40 to 49, 50 to 59, 60 to 69, and 70 to 79 years), the mean blood pressure increased and the blood pressure distribution within an age class approached the normal distribution with advancing age in both sexes (Figure VI in the online-only Data Supplement). Besides sex, age, and BMI, although modest in the explained variance (R^2), alcohol intake and smoking correlated with blood pressure increase (R^2=0.002 to 0.015) and decrease (R^2=0.003 to 0.012), respectively, in the studied population (Table V in the online-only Data Supplement).

We calculated R^2 for blood pressure explained by BMI and that explained by the risk score on combining the 7 SNP data within each age class separately by sex on the basis of the results for the 3 population cohorts (online-only Data Supplement). In both sexes, BMI contributed to blood pressure variance to a larger extent at 30 to 49 years of age than at ≥50 and years of age. The cumulative genetic effects (represented by 7 SNPs) were almost unchanged throughout the age classes in women, whereas they were the highest at 40 to 49 years of age and tended to decrease with age (in particular, R^2<0.0001 at 70 to 79 years of age) in men (Figure 3 and Figure VII in the online-only Data Supplement). There was a difference in the cumulative genetic effects on DBP at ≥70 years of age between the sexes (P=0.046, not adjusted for multiple comparisons; Table VI in the online-only Data Supplement). We then examined the modulation of genetic associations by sex, age, and BMI but obtained no conclusive evidence of interaction after the correction of multiple testing (see text and Tables VI through VIII in the online-only Data Supplement).

Discussion

By this multistaged replication study involving a total of 25826 Japanese subjects, we have confirmed genetic associations at 7 loci with blood pressure and/or hypertension in a population of non-European ancestry. In addition to 5 (of 7) loci—*FGF5*, *MTHFR*, *CYP17A1-CNNM2*, *ATP2B1*, and *CSK-ULK3*—that have genome-wide significance in Europeans, our study replicated 2 loci, *CASZ1* and *ITGA9*, for which suggestive evidence of association was previously reported.[6,7] Most of the replicated variants were associated concordantly with both blood pressure traits (SBP and DBP) and dichotomous hypertension, except for *MTHFR* and *ITGA9*, for which either blood pressure or hypertension association was rather pronounced in the Japanese (Tables 2 and 3 and Figure VA and VB in the online-only Data Supplement). Furthermore, despite the small number of SNP loci described here (7 SNPs) and the small proportion of blood pressure variation explained by these SNPs (R^2=0.005), the conjoint effect of multiple risk alleles on blood pressure levels and hypertension is sufficient to increase the cardiovascular risk (Figure 2A and 2B). Moreover, while examining the impacts of confounding factors on the genetic associations with blood pressure and/or hypertension, we detected a trend for male-specific interactions between age and the genetic associations; ie, the SNP–blood pressure association is influenced by age in a sex-specific manner, at least for the loci tested in the present study (Figure 3). Although the interaction has not been proven to be statistically significant, this seems to be in good agreement with the previous studies,[4,9] which documented the modulation of genetic effects on blood pressure by sex and age.

There is substantial overlap of common genetic variants influencing blood pressure and the risk of hypertension between the Japanese (or East Asians) and Europeans, whereas some ethnic heterogeneity is likely to exist in the effect sizes for each risk allele. In the Japanese, the strongest association was observed for *FGF5*. Although this locus shows a robust and significant association in Europeans,[7] we detected significant cross-population heterogeneity (P=0.01 for SBP) in the strength of association with blood pressure; the SBP and DBP effects (on the SD scale) were >1.4 times larger in the Japanese than in Europeans (Figure VA in the online-only Data Supplement). Furthermore, the OR for hypertension significantly differed at *FGF5* between the populations (OR, 1.29 and 1.10 in the Japanese and Europeans, respectively; Figure VB in the online-only Data Supplement). Despite a less conserved linkage disequilibrium block in the Japanese than in Europeans near rs16998073 (3.4 kb upstream of *FGF5* on 4q21; Figure VIII in the online-only

Data Supplement), we could still detect strong association signals at this locus across the 3 Japanese cohorts (Figure IV in the online-only Data Supplement). The *FGF5* gene itself is a promising candidate because it encodes a member of the fibroblast growth factor family, and the protein (fibroblast growth factor 5) is noted for its effects in promoting angiogenesis in the heart.[16]

Similarly, significant cross-population heterogeneity was observed at *CASZ1*. Although suggestive evidence of association with SBP has been identified at this locus in Europeans (β=0.53 and $P=4.8\times10^{-6}$),[6] the genetic association was stronger for all 3 traits in the Japanese (β=1.08 and $P=2.2\times10^{-8}$ for SBP, β=0.79 and $P=4.9\times10^{-12}$ for DBP, OR=1.18 and $P=3.0\times10^{-7}$ for hypertension; Tables 2 and 3). The associated SNP, rs880315, is located in the intron of the *CASZ1* gene. Although little is known about its molecular function, *CASZ1* (encoding the castor homolog 1, zinc finger) is reportedly a cell survival gene that controls apoptosis and tumor formation.[17] One mechanism linking *CASZ1* to vascular or heart dysfunction would be inflammatory responses involving cell adhesion, permeability, and apoptosis.

Genetic associations were confirmed at 5 other loci—*MTHFR*, *CYP17A1-CNNM2*, *ATP2B1*, *CSK-ULK3*, and *ITGA9*—in the Japanese, with some interethnic fluctuation in the effect sizes. On the other hand, several candidate loci previously reported by GWA studies of Europeans[6,7] did not pass our stage 1 screening. This could reflect the limited power to detect the modest effect size (average power=0.62 in the stage 1 panel; Table I in the online-only Data Supplement) and/or the lack of (or weaker) association among the Japanese subjects due partly to differences in linkage disequilibrium patterns between the ethnic groups.

The strengths of the present study are that we used 3 independent cohorts of the Japanese population; that the study subjects were enrolled from regions of Japan with no strong population stratification[18]; and that the subjects totaled 25 826 (49.7% female), belonging to a wide range of age groups (18 to 97 years). In the population at large, blood pressure (in particular, SBP) tends to increase and the prevalence of hypertension becomes higher with advancing age, when prominent sex differences are widely recognizable.[19] When we examined modulation of SNP–blood pressure association by sex and age (Figure 3 and Table VI in the online-only Data Supplement), we unexpectedly found that R^2 for blood pressure, explained by a combination of SNPs or by an individual SNP, fluctuates with age in a sex-specific manner. The possibility of this gene-age-sex interaction is worthy of note but should be carefully interpreted because it has not attained a conclusive level of statistical significance after adjustment for multiple testing. For both SBP and DBP, R^2 explained by the 7 SNPs was highest at 40 to 49 years of age, when R^2 explained by BMI was also the highest, and tended to decrease at older ages in men but was almost stable in women. Older subjects are more likely to have hypertension and to be taking antihypertensive drugs than younger adults. While analyzing blood pressure, we adjusted for the use of antihypertensive therapy by adding 15 and 10 mm Hg to SBP and DBP, respectively, as has been carried out by previous studies to reduce bias and to improve statistical power.[7,15] It is possible that SBP and DBP measures thus imputed as quantitative traits incorporate some bias in the older age groups, thereby leading to underestimation of R^2. This speculation, however, cannot explain the sexual dimorphism detected in the possible interactions of the genetic associations with age. That is, considering that age-related changes in R^2 explained by BMI were similarly observable in both sexes, the male-specific fluctuations in R^2 explained by the 7 SNPs cannot be attributed to simple bias in blood pressure and warrant further investigation.

We acknowledge that there are several limitations in the present study. Although we recruited a major part of the study subjects (stages 2a and 2b) from 3 cohorts of the Japanese population, there is some intercohort diversity in the range of age and treatment profiles (Table 1). Given the appreciable influence of age on the genetic association and the potential age-related bias in adjustment for the use of antihypertensive therapy, these factors could reduce the power of detecting the association of genetic variants with modest effects. In this respect, larger sample sizes (particularly in the stage 1 screening) are required to thoroughly examine modest associations at the loci previously identified by the GWA studies of Europeans[6,7] in the Japanese; the power calculation is given in Figure II and Table I and explained in the text of the online-only Data Supplement. Moreover, because cause-and-effect relationships cannot be inferred by cross-sectional studies, longitudinal studies need to be performed to validate sex- and age-specific modulation of blood pressure associations and to clarify the underlying mechanisms.

Conclusions

We have confirmed that 7 loci are associated with blood pressure and/or hypertension in the Japanese. These loci can guide our fine-mapping efforts to pinpoint causal variants and causal genes with the integration of multiethnic results.

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Disclosures

None.

References

1. Lawes CM, Vander Hoorn S, Rodgers A, for the International Society of Hypertension. Global burden of blood-pressure-related disease, 2001. *Lancet*. 2008;371:1513–1518.

2. Prospective Studies Collaboration. Age-specific relevance of usual blood pressure to vascular mortality: a meta-analysis of individual data for one million adults in 61 prospective studies. *Lancet*. 2002;360:1903–1913.
3. Pilia G, Chen WM, Scuteri A, Orrú M, Albai G, Dei M, Lai S, Usala G, Lai M, Loi P, Mameli C, Vacca L, Deiana M, Olla N, Masala M, Cao A, Najjar SS, Terracciano A, Nedorezov T, Sharov A, Zonderman AB, Abecasis GR, Costa P, Lakatta E, Schlessinger D. Heritability of cardiovascular and personality traits in 6,148 Sardinians. *PLoS Genet*. 2006; 2:e132.
4. Seda O, Tremblay J, Gaudet D, Brunelle PL, Gurau A, Merlo E, Pilote L, Orlov SN, Boulva F, Petrovich M, Kotchen TA, Cowley AW Jr, Hamet P. Systematic, genome-wide, sex-specific linkage of cardiovascular traits in French Canadians. *Hypertension*. 2008;51:1156–1162.
5. Wellcome Trust Case Control Consortium. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature*. 2007;447:661–678.
6. Levy D, Ehret GB, Rice K, Verwoert GC, Launer LJ, Dehghan A, Glazer NL, Morrison AC, Johnson AD, Aspelund T, Aulchenko Y, Lumley T, Köttgen A, Vasan RS, Rivadeneira F, Eiriksdottir G, Guo X, Arking DE, Mitchell GF, Mattace-Raso FU, Smith AV, Taylor K, Scharpf RB, Hwang SJ, Sijbrands EJ, Bis J, Harris TB, Ganesh SK, O'Donnell CJ, Hofman A, Rotter JI, Coresh J, Benjamin EJ, Uitterlinden AG, Heiss G, Fox CS, Witteman JC, Boerwinkle E, Wang TJ, Gudnason V, Larson MG, Chakravarti A, Psaty BM, van Duijn CM. Genome-wide association study of blood pressure and hypertension. *Nat Genet*. 2009;41:677–687.
7. Newton-Cheh C, Johnson T, Gateva V, Tobin MD, Bochud M, Coin L, Najjar SS, Zhao JH, Heath SC, Eyheramendy S, Papadakis K, Voight BF, Scott LJ, Zhang F, Farrall M, Tanaka T, Wallace C, Chambers JC, Khaw KT, Nilsson P, van der Harst P, Polidoro S, Grobbee DE, Onland-Moret NC, Bots ML, Wain LV, Elliott KS, Teumer A, Luan J, Lucas G, Kuusisto J, Burton PR, Hadley D, McArdle WL; Wellcome Trust Case Control Consortium, Brown M, Dominiczak A, Newhouse SJ, Samani NJ, Webster J, Zeggini E, Beckmann JS, Bergmann S, Lim N, Song K, Vollenweider P, Waeber G, Waterworth DM, Yuan X, Groop L, Orho-Melander M, Allione A, Di Gregorio A, Guarrera S, Panico S, Ricceri F, Romanazzi V, Sacerdote C, Vineis P, Barroso I, Sandhu MS, Luben RN, Crawford GJ, Jousilahti P, Perola M, Boehnke M, Bonnycastle LL, Collins FS, Jackson AU, Mohlke KL, Stringham HM, Valle TT, Willer CJ, Bergman RN, Morken MA, Döring A, Gieger C, Illig T, Meitinger T, Org E, Pfeufer A, Wichmann HE, Kathiresan S, Marrugat J, O'Donnell CJ, Schwartz SM, Siscovick DS, Subirana I, Freimer NB, Hartikainen AL, McCarthy MI, O'Reilly PF, Peltonen L, Pouta A, de Jong PE, Snieder H, van Gilst WH, Clarke R, Goel A, Hamsten A, Peden JF, Seedorf U, Syvänen AC, Tognoni G, Lakatta EG, Sanna S, Scheet P, Schlessinger D, Scuteri A, Dörr M, Ernst F, Felix SB, Homuth G, Lorbeer R, Reffelmann T, Rettig R, Völker U, Galan P, Gut IG, Hercberg S, Lathrop GM, Zelenika D, Deloukas P, Soranzo N, Williams FM, Zhai G, Salomaa V, Laakso M, Elosua R, Forouhi NG, Völzke H, Uiterwaal CS, van der Schouw YT, Numans ME, Matullo G, Navis G, Berglund G, Bingham SA, Kooner JS, Connell JM, Bandinelli S, Ferrucci L, Watkins H, Spector TD, Tuomilehto J, Altshuler D, Strachan DP, Laan M, Meneton P, Wareham NJ, Uda M, Jarvelin MR, Mooser V, Melander O, Loos RJ, Elliott P, Abecasis GR, Caulfield M, Munroe PB. Genome-wide association study identifies eight loci associated with blood pressure. *Nat Genet*. 2009;41:666–676.
8. Wang X, Poole JC, Treiber FA, Harshfield GA, Hanevold CD, Snieder H. Ethnic and gender differences in ambulatory blood pressure trajectories: results from a 15-year longitudinal study in youth and young adults. *Circulation*. 2006;114:2780–2787.
9. Shi G, Gu CC, Kraja AT, Arnett DK, Myers RH, Pankow JS, Hunt SC, Rao DC. Genetic effect on blood pressure is modulated by age: the Hypertension Genetic Epidemiology Network Study. *Hypertension*. 2009;53:35–41.
10. Pausova Z, Gaudet D, Gossard F, Bernard M, Kaldunski ML, Jomphe M, Tremblay J, Hudson TJ, Bouchard G, Kotchen TA, Cowley AW, Hamet P. Genome-wide scan for linkage to obesity-associated hypertension in French Canadians. *Hypertension*. 2005;46:1280–1285.
11. Tsuchihashi-Makaya M, Serizawa M, Yanai K, Katsuya T, Takeuchi F, Fujioka A, Yamori Y, Ogihara T, Kato N. Gene-environmental interaction regarding alcohol-metabolizing enzymes in the Japanese general population. *Hypertens Res*. 2009;32:207–213.
12. Nanri A, Yoshida D, Yamaji T, Mizoue T, Takayanagi R, Kono S. Dietary patterns and C-reactive protein in Japanese men and women. *Am J Clin Nutr*. 2008;87:1488–1496.
13. Ogihara T, Kikuchi K, Matsuoka H, Fujita T, Higaki J, Horiuchi M, Imai Y, Imaizumi T, Ito S, Iwao H, Kario K, Kawano Y, Kim-Mitsuyama S, Kimura G, Matsubara H, Matsuura H, Naruse M, Saito I, Shimada K, Shimamoto K, Suzuki H, Takishita S, Tanahashi N, Tsuchihashi T, Uchiyama M, Ueda S, Ueshima H, Umemura S, Ishimitsu T, Rakugi H. The Japanese Society of Hypertension guidelines for the management of hypertension (JSH 2009). *Hypertens Res*. 2009;32:3–107.
14. Takeuchi F, Serizawa M, Yamamoto K, Fujisawa T, Nakashima E, Ohnaka K, Ikegami H, Sugiyama T, Katsuya T, Miyagishi M, Nakashima N, Nawata H, Nakamura J, Kono S, Takayanagi R, Kato N. Confirmation of multiple risk Loci and genetic impacts by a genome-wide association study of type 2 diabetes in the Japanese population. *Diabetes*. 2009;58: 1690–1699.
15. Tobin MD, Sheehan NA, Scurrah KJ, Burton PR. Adjusting for treatment effects in studies of quantitative traits: antihypertensive therapy and systolic blood pressure. *Stat Med*. 2005;24:2911–2935.
16. Vatner SF. FGF induces hypertrophy and angiogenesis in hibernating myocardium. *Circ Res*. 2005;96:705–707.
17. Yuan ZR, Wang R, Solomon J, Luo X, Sun H, Zhang L, Shi Y. Identification and characterization of survival-related gene, a novel cell survival gene controlling apoptosis and tumorigenesis. *Cancer Res*. 2005;65: 10716–10724.
18. Yamaguchi-Kabata Y, Nakazono K, Takahashi A, Saito S, Hosono N, Kubo M, Nakamura Y, and Kamatani N. Japanese population structure, based on SNP genotypes from 7003 individuals compared to other ethnic groups: effects on population-based association studies. *Am J Hum Gene*. 2008;83:445–456.
19. Safar ME, Smulyan H. Hypertension in women. *Am J Hypertens*. 2004;17:82–87.

CLINICAL PERSPECTIVE

A substantial contribution of genetic factors to hypertension or high blood pressure has been documented by a number of epidemiological studies. However, identifying common variants affecting blood pressure using genome-wide association studies has proved challenging compared with the success of genome-wide association studies of other common diseases. Recently, 2 consortium-based genome-wide association studies have successfully identified robust and significant associations of common variants with systolic and diastolic blood pressures in populations of European descent, warranting further investigation in populations of non-European descent. In the present study, we examined the associations at 27 loci reported by the genome-wide association studies on Europeans in a screening panel of Japanese subjects (n=1526) and chose 11 loci showing association signals (1-tailed test in the screening, $P<0.3$) for an extensive replication study with a follow-up panel of 3 Japanese general population cohorts (n $\leq$24 300). Significant associations were replicated for 7 loci—*CASZ1*, *MTHFR*, *ITGA9*, *FGF5*, *CYP17A1-CNNM2*, *ATP2B1*, and *CSK-ULK3*—with any or all of the following 3 traits: systolic blood pressure ($P=1.4\times10^{-14}$ to 0.05), diastolic blood pressure ($P=1.9\times10^{-12}$ to 0.05), and hypertension ($P=2.0\times10^{-14}$ to 0.006; odds ratio, 1.10 to 1.29). Besides confirming the association at 7 loci in the Japanese, we have detected the potential presence of gene-age-sex interactions, emphasizing the importance of such interactions in the molecular approach to hypertension genetics.