Blood Pressure Levels and Bleeding Events During Antithrombotic Therapy

The Bleeding With Antithrombotic Therapy (BAT) Study

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Background and Purpose—A prospective, multicenter, observational cohort study was conducted to clarify the association between major bleeding events and blood pressure (BP) levels during follow-up before development of bleeding events in antithrombotic users.

Methods—A total of 4009 patients taking oral antithrombotic agents for cardiovascular or cerebrovascular diseases (2728 men, 69±10 years old) were followed. Changes in systolic and diastolic BPs between entry and the last clinic visit before intracranial hemorrhage (ICH) or extracranial hemorrhage were assessed.

Results—Over a median follow-up of 19 months, ICH developed in 31 patients and extracranial hemorrhage developed in 77. Entry BP levels were similar among patients with ICH, those with extracranial hemorrhage, and those without hemorrhagic events. Both systolic BP and diastolic BP were relatively high during follow-up as compared with the levels at entry in patients with ICH, whereas they showed plateaus in patients with extracranial hemorrhage and patients without hemorrhagic events. Average systolic BP levels between 1 and 6 months (hazard ratio, 1.45; 95% CI, 1.08 to 1.92 per 10-mm Hg increase) and between 7 and 12 months (hazard ratio, 1.47; 95% CI, 1.05 to 2.01) as well as average diastolic BP levels between 7 and 12 months (hazard ratio, 2.05; 95% CI, 1.15 to 3.62) were independently associated with development of ICH after adjustment for established ICH predictors. The optimal cutoff BP level to predict impending risk of ICH was ≥130/81 mm Hg using receiver operating characteristic curve analysis.

Conclusions—An increase in BP levels during antithrombotic medication was positively associated with development of ICH, suggesting the importance of adequate BP control for avoiding ICH. BP levels did not appear to be associated with extracranial hemorrhage. (***Stroke*. 2010;41:1440-1444.**)

Key Words: anticoagulation ■ antiplatelet therapy ■ hypertension ■ intracerebral hemorrhage ■ stroke

Antithrombotic therapy is regarded as an essential primary and secondary preventive strategy for cardiovascular diseases and stroke.[1,2] However, bleeding events are inevitable complications of this therapy; in particular, intracranial hemorrhage (ICH) is a typical life-threatening event.[3] Carefully regulated warfarin therapy to international normalized ratios between 2 and 3 doubles the risk of ICH, and aspirin increases the risk by approximately 40%.[4]

Hypertension is a firmly established risk factor for ICH in the general population[5] as well as in warfarin users.[4] In the Perindopril Protection Against Recurrent Stroke Study (PROGRESS), in which 72% of enrolled patients with stroke were receiving antiplatelets and 10% were receiving anticoagulants, ICH was reduced by half after mean blood pressure (BP) -lowering by 9/4 mm Hg.[6] Thus, adequate antihypertensive therapy seems to prevent ICH during antithrombotic therapy. This raises an essential issue: whether antithrombotic users who finally developed ICH and other bleeding events had high BP levels throughout follow-up as well as how such patients' BP levels changed during follow-up.

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To determine the incidence and severity of bleeding complications in patients with cardiovascular diseases and stroke treated with oral antithrombotic therapy in Japan, a prospective, multicenter, observational study (the Bleeding with Antithrombotic Therapy [BAT] Study) was conducted. In its initial report of the overall results, adding antiplatelets to warfarin or single antiplatelet therapy doubled the risk of life-threatening or major bleeding events.[7] Here, the association between these patients' BP levels during follow-up and development of bleeding events was determined.

Patients and Methods

The BAT Study was a prospective, multicenter, observational cohort study on the incidence and severity of bleeding complications in antithrombotic users. A total of 4009 patients (2728 men, 69±10 years [mean±SD]) who were taking oral antiplatelet agents or warfarin for cardiovascular or cerebrovascular diseases were consecutively enrolled from 19 stroke and cardiovascular centers that were balanced regionally in Japan and observed for 2 to 30 months between October 2003 and March 2006. The study protocol, inclusion/exclusion criteria, and general results were published previously.[7] The medical ethics review boards of the participating institutes approved the study protocol, and all patients provided written informed consent.

Based on bleeding events during follow-up, the patients were divided into 3 groups: an "ICH group" for the patients developing any symptomatic ICH; an "extracranial hemorrhage (ECH) group" for those developing a life-threatening or major bleeding event other than ICH; and a "non-H group" for those without any life-threatening or major bleeding event. Bleeding events were classified according to the definition by the Management of ATherothrombosis with Clopidogrel in High-risk patients with recent transient ischemic attack or ischemic stroke study (MATCH).[8] Briefly, life-threatening bleeding was defined as: any fatal bleeding event; a drop in hemoglobin of ≥50 g/L; hemorrhagic shock; symptomatic ICH; or transfusion of ≥4 U of red blood cells. Major bleeding was defined as significantly disabling, severe intraocular bleeding, or transfusion of ≤3 U of red blood cells. Secondary hemorrhagic transformation of an ischemic stroke was not regarded as a bleeding event. When the patients developed a life-threatening or major bleeding event, observation was discontinued.

Comorbidities (ischemic and hemorrhagic stroke, heart disease, neoplasms, and liver cirrhosis) and cardiovascular risk factors (hypertension, diabetes mellitus, hypercholesterolemia, hypocholesterolemia [serum total cholesterol <130 mg/dL on enrollment], current or previous smoking habit, and alcohol consumption ≥2 drinks per day) listed in this study were the same as those in the previous study.[7] Follow-up evaluations were normally performed every month; each time, BP was measured using a mercury sphygmomanometer.

Statistical Methods

All analyses were performed using JMP 7 statistical software (SAS Institute Inc, Cary, NC). Average levels of systolic and diastolic BPs (SBP and DBP, respectively) between 1 and 6 months, between 7 and 12 months, and after 13 months as well as the levels at entry were assessed for the Cox proportional hazards regression analysis. BP levels at the last clinic visit of the observation period (the last visit before bleeding events for the ICH and ECH groups) and the average BP levels of all the follow-up measurements except for the levels at entry and at the last visit were assessed for the annual incidence and 95% CIs of ICH and the receiver operating characteristic (ROC) curves analysis. To compare baseline clinical characteristics and BP levels among the ICH, ECH, and Non-H groups, 1-way factorial analysis of variance with post hoc comparison by Dunnett test (with Non-H patients as control subjects) was used for continuous variables, and the χ^2 test was used for categorical variables. To examine the associations of BP levels and their changes with the development of ICH, a Cox proportional hazards regression analysis was performed using a forced entry method of established ICH predictors, including sex, age, hypertension, diabetes mellitus, current or previous smoking habit, alcohol consumption, prior cerebrovascular disease, and use of warfarin. Goodness of fit of the statistical model was tested using the likelihood ratio in the Whole Model Test and Akaike information criterion. Finally, the optimal cutoff BP levels to predict impending development of ICH (in other words, to predict the last clinic visit before ICH) were determined using ROC curves based on all the BP measurements during follow-up. A probability value <0.05 was considered statistically significant.

Results

Of 4009 enrolled patients, 1891 (47.2%) were taking single antiplatelet agents, 349 (8.7%) were taking dual antiplatelet agents, 1298 (32.4%) were taking warfarin, and 471 (11.7%) were taking warfarin plus antiplatelet agents. The main antiplatelet agents used in the enrolled patients were described previously.[7] Briefly, aspirin monotherapy, ticlopidine monotherapy, and aspirin plus ticlopidine were the major choice for both antiplatelet users (1340, 394, and 220 patients, respectively) and warfarin plus antiplatelets users (336, 69, and 49 patients, respectively). At entry, the median international normalized ratio was 1.97 (interquartile range, 1.69 to 2.33) in warfarin users (taking warfarin alone or warfarin plus antiplatelets).

During the median observation period of 19 months (interquartile range, 13 to 23 months), 108 life-threatening or major bleeding events, including 31 ICH and 77 ECH, occurred. In warfarin users, the median international normalized ratio at entry was 2.06 (interquartile range, 1.95 to 2.30) in the ICH group, 2.06 (1.65 to 2.46) in the ECH group, and 1.96 (1.69 to 2.33) in the Non-H group (P=0.149); and the median international normalized ratio at the last visit before bleeding events or on the day of the event was 2.28 (1.74 to 2.68) in the ICH group and 2.24 (1.75 to 3.06) in the ECH group (P=0.993). Among the 3 groups, observation period (P<0.001), age (P=0.003), use of warfarin (P=0.002), and neoplasm (P=0.013) were significantly different (Table 1).

Figure 1 shows the time courses of the BP levels. Both SBP and DBP levels at entry were similar among the 3 groups (Table 1). During follow-up, both SBP and DBP were relatively high as compared with the levels at entry in the ICH group, and they plateaued in the ECH and Non-H groups. BP levels were not significantly different among the 3 groups in any BP measurements.

The association of BP with the development of ICH was determined after adjustment for sex, age, hypertension, diabetes mellitus, current or previous smoking habit, alcohol consumption, prior cerebrovascular disease, and use of warfarin (Table 2). Average SBP levels between 1 and 6 months (hazard ratio [HR], 1.45; 95% CI, 1.08 to 1.92 per 10-mm Hg increase) and between 7 and 12 months (HR, 1.47; 95% CI, 1.05 to 2.01) as well as average DBP levels between 7 and 12 months (HR, 2.05; 95% CI, 1.15 to 3.62) were independently associated with ICH. The probability value of likelihood ratio in the Whole Model Test after multivariate adjustment was 0.055 for SBP at entry, 0.007 for average SBP between 1 and 6 months, 0.014 for average SBP between 7 and 12 months, 0.114 for average SBP after 13 months, 0.066 for DBP at entry, 0.046 for average DBP between 1 and 6 months, 0.010

Table 1. Patients' Baseline Clinical Characteristics

	ICH	ECH	Non-H	P
Patient no.	31	77	3901	
Observation period, months	11 (5–14)	11 (6–14)	19 (14–23)	<0.001
Age, years	73±7	71±10	69±10	0.003
Male	81%	75%	69%	0.173
Use of warfarin*	61%	61%	44%	0.002
Comorbidities				
Ischemic stroke	68%	44%	55%	0.060
Hemorrhagic stroke	6%	1%	2%	0.122
Heart disease, arrhythmia	77%	74%	67%	0.217
Neoplasm	19%	12%	7%	0.013
Liver cirrhosis	6%	4%	2%	0.197
Risk factors				
Hypertension	65%	57%	61%	0.746
Diabetes mellitus	26%	34%	26%	0.296
Hypercholesterolemia	36%	32%	42%	0.173
Hypocholesterolemia	3%	1%	1%	0.152
Smoking habit, current	19%	10%	14%	0.269
Smoking habit, previous	29%	47%	36%	
Alcohol consumption	10%	6%	5%	0.413
SBP at entry, mm Hg	134.6±13.2	130.8±18.5	132.5±17.9	0.597
DBP at entry, mm Hg	74.8±12.3	74.5±10.4	75.6±11.0	0.672

Data are medians (interquartile range) for the observation period, means±SD for age and BP, and percent of patients for others.

*Taking warfarin alone or warfarin plus antiplatelets.

for average DBP between 7 and 12 months, and 0.117 for average DBP after 13 months. Thus, SBP between 1 and 6 months, SBP between 7 and 12 months, and DBP between 7 and 12 months showed relatively good fitness. Akaike information criterion was 446.4, 438.1, 326.4, and 204.4 for each SBP measurement and 447.0, 443.3, 325.6, and 204.5 for each DBP measurement, respectively. Based on Akaike information criterion, SBP and DBP after 13 months were better than other BP measurements in regard to goodness of fit.

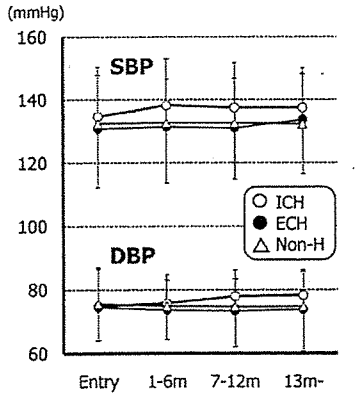

Figure 1. Time courses of BP. Average levels of SBP and DBP between 1 and 6 months, between 7 and 12 months, and after 13 months as well as the levels at entry are plotted. ICH indicates patients developing any symptomatic ICH; ECH, patients developing a life-threatening or major bleeding event other than ICH; Non-H, patients without any life-threatening or major bleeding event. All patients are included at entry and during 1 and 6 months; 21 patients with ICH, 53 patients with ECH, and 3293 Non-H patients are included during 7 and 12 months; and 13 patients with ICH, 30 patients with ECH, and 2936 Non-H patients are included after 13 months.

Table 2. Multivariate-Adjusted HR and 95% CI of BP Parameters for Development of ICH*

	HR	95% CI	P
SBP			
Level at entry	1.09	0.88–1.34	0.435
Mean level between 1 and 6 months	1.45	1.08–1.92	0.013
Mean level between 7 and 12 months	1.47	1.05–2.01	0.026
Mean level after 13 months	1.29	0.93–1.76	0.120
DBP			
Level at entry	0.97	0.68–1.39	0.880
Mean level between 1 and 6 months	1.28	0.78–2.13	0.337
Mean level between 7 and 12 months	2.05	1.15–3.62	0.016
Mean level after 13 months	1.50	0.89–2.53	0.126

*Per 10-mm Hg increase. Adjusted for sex, age, hypertension, diabetes mellitus, current or previous smoking habit, alcohol consumption, prior cerebrovascular disease, and use of warfarin.

Because the observation was discontinued within 6 months or within 12 months for many patients, especially for those with ICH and ECH, the following analyses were performed using BP levels at the last clinic visit and the average BP levels of all the follow-up measurements except for the levels at entry and at the last visit. At the last visit, both SBP and DBP were higher in the ICH group than in the Non-H group (141.7±13.6/81.3±10.3 mm Hg versus 132.4±17.8/74.7±10.9 mm Hg, P=0.011 for SBP and P=0.003 for DBP). Figure 2 shows annual incidence of ICH according to BP levels. ICH risk increased linearly as both SBP and DBP levels at the last clinic visit increased; the risk did not increase linearly as BP levels at entry or those during follow-up increased.

To predict the impending development of ICH, the optimal cutoff SBP level determined using ROC curves was ≥130 mm Hg with a sensitivity of 89.3%, specificity of

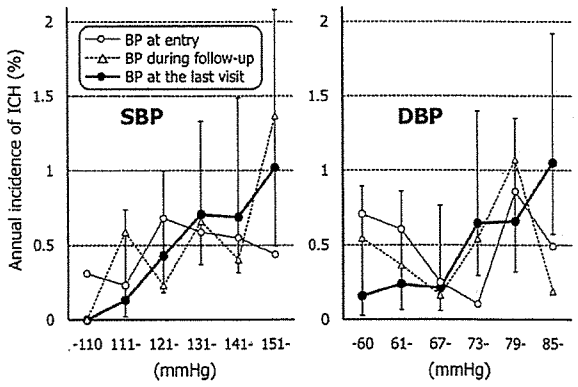

Figure 2. Annual incidence of ICH according to SBP and DBP levels. Bars indicate 95% CI for BP at the last clinic visit. "BP during follow-up" means average BP levels of all the follow-up measurements except for the levels at entry and at the last visit.

41.8%, and an area under the ROC curve of 0.659; the optimal cutoff DBP level was ≥81 mm Hg with a sensitivity of 53.6%, specificity of 74.2%, and an area under the ROC curve of 0.676. Both SBP3 ≥130 mm Hg (OR, 6.23; 95% CI, 2.16 to 26.35; $P<0.001$) and DBP3 ≥81 mm Hg (OR, 3.49; 95% CI, 1.64 to 7.52; $P=0.001$) were independently associated with ICH after adjustment for the 8 established ICH predictors.

Discussion

A major new finding of the present observational study was that BP levels during the follow-up, but not the level at entry, were independently associated with the development of ICH. In particular, ICH risk increased linearly as BP levels at the last clinic visit increased. The estimated cutoff BP level to predict impending risk of ICH was ≥130/81 mm Hg. BP levels did not appear to be associated with major systemic (excluding intracranial) bleeding events.

Hypertension is an established modifiable risk factor for ICH during warfarin therapy along with intensity of anticoagulation, concomitant use of antiplatelets, and smoking and heavy drinking habits.[4] However, major trials involving anticoagulant users failed to show entry BP level as a predictor for major bleeding events.[9–11] To resolve the contradiction, we designed the present study, which assessed BP levels during follow-up. The present antithrombotic users developing ICH had approximately 2 to 4 mm Hg higher entry SBP than those without bleeding events, which was not statistically significant. However, their SBP and DBP increased by an average of approximately 4 mm Hg at the follow-up as compared with at entry, and this increase may trigger ICH. Such an increase might result from careless BP management or resistance to antihypertensive therapy. Regardless of the cause, avoidance of a BP increase would lessen the risk for ICH.

Based on differences in average BP levels at the last visit between the ICH group and the other 2 groups, we hypothesized that the cutoff SBP level to predict impending development of ICH was roughly between 132 and 142 mm Hg, and the cutoff DBP level was roughly between 75 and 81 mm Hg. After ROC curve analyses, 130/81 mm Hg appears to be the cutoff level. Although the statistical power judged from the area under the ROC curve is not strong, this cutoff level seems to be reasonable, because recent guidelines from the European Society of Hypertension and the European Society of Cardiology and those from the Japanese Society of Hypertension advocated <130/80 mm Hg as the target BP level in diabetics and in high- or very-high-risk patients.[12,13] Real target BP levels during antithrombotic therapy should be determined by systematic comparative trials.

Combination therapy with antithrombotics and antihypertensives appears to be preventive for ICH. In the interim report of the Secondary Prevention of Small Subcortical Strokes (www.sps3.org/), in which SBP was lowered to <149 mm Hg or <130 mm Hg, risk of ICH was less than expected in patients with stroke taking aspirin alone or aspirin plus clopidogrel (personal communication). Success in reducing ICH in PROGRESS, in which 82% of enrolled patients were receiving antithrombotics, was reviewed.[6] On the other hand, an angiotensin receptor blocker, telmisartan, did not reduce the risk of ICH for antiplatelet users who recently had ischemic stroke in the Prevention Regimen for Effectively Avoiding Second Strokes (PRoFESS) study (HR, 0.81; 95% CI, 0.63 to 1.05)[14]; the relatively small number of patients developing ICH may be a reason for this failure to show an effect.

Major systemic (not intracranial) bleeding events developed under identical BP levels as those in our patients without major bleeding events. This indicates that hypertensive damage to gastrointestinal, dermal, and other systemic circulations is milder than the damage to cerebral circulation. Preventive strategies other than antihypertensives, including proton pump inhibitors and H2 receptor antagonists, appear to be promising for reducing gastrointestinal bleeding.[15,16]

The limitations of the present study include the relatively short duration of the observation period and the small numbers of bleeding events as a result, which may affect the statistical results and made it difficult to perform subanalyses for patients with different clinical backgrounds and different antithrombotic regimens. Second, information on patients' antihypertensive therapy was not given. Third, clopidogrel, a universal antiplatelet agent, was not used in our patients because the agent was approved for use in Japan in 2006, after the study was finished. Finally, data of many patients were not included in the analysis of the follow-up BP measurements during 7 and 12 months and after 13 months partly because of early discontinuance of the observation due to bleeding events. To overcome this limitation and to introduce a message that BP levels at the last clinic visit are important for ICH risk, we used the BP levels at the last visit for some analyses, including the ROC. However, it is not originally appropriate to use the last available measurement as a predictor of a bleeding event in a prospective study.

Because ischemic events are much more common than bleeding events, the use of antithrombotic agents has been increasing. The present study suggests that one should be careful to avoid BP elevations in antithrombotic users, and it is important to lower their BP adequately to avoid ICH.

Appendix

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Disclosures

None.

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Confirmation of an Association of Single-Nucleotide Polymorphism rs1333040 on 9p21 With Familial and Sporadic Intracranial Aneurysms in Japanese Patients

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Background and Purpose—Genetic factors are important determinants of intracranial aneurysm (IA). Recently, a multinational, genome-wide association study identified 3 loci associated with IA, located on 2q (rs700651), 8q (rs10958409), and 9p (rs1333040 and rs10757278). The aim of this study was to evaluate these associations.

Methods—Familial and sporadic cases were investigated. Familial cases, consisting of 96 subjects with IA, and 46 subjects of unknown status from 31 pedigrees were analyzed with the transmission disequilibrium test and linkage analysis. Associations of single-nucleotide polymorphisms (SNPs) with IA were tested in 419 sporadic IA cases and in 408 control subjects. Sequencing of *CDKN2A*, *CDKN2B*, and *CDKN2BAS* revealed additional SNPs, and their associations with IA were also tested.

Results—The transmission disequilibrium test revealed associations of 2 SNPs, rs700651 (P=0.036) and rs1333040 (P=0.002), with familial IA. Analysis of SNPs in sporadic cases revealed an allelic association of rs1333040 with IA (odds ratio=1.28; 95% CI, 1.04–1.57; P=0.02) but failed to show associations of rs10757278 and rs496892 with IA. We sequenced 3 candidate genes; *CDKN2A*, *CDKN2B*, and *CDKN2BAS*. All 6 index cases from IA families had the rs1333040-T allele and SNPs (rs10965215, rs10120688, and rs7341791) in *CDKN2BAS*. None of these SNPs had linkage disequilibrium with rs1333040 and was associated with IA.

Conclusions—A region between introns 7 and 15 of *CDKN2BAS* carrying the rs1333040-T allele may confer risk for IA. (***Stroke.* 2010;41:1138-1144.**)

Key Words: genetics ■ intracranial aneurysm ■ association study ■ *CDKN2BAS* ■ single-nucleotide polymorphisms

Subarachnoid hemorrhage (SAH) is fatal in ≈50% of cases, and significant disability is caused in ≈30% of cases, despite the diagnostic and therapeutic developments of the past few decades.[1,2] In Japan, the total annual mortality rate for SAH was estimated to be 22.5 per 100 000 person-years.[3] Rupture of intracranial aneurysm (IA) accounts for >90% of SAH cases.[2] Both environmental and genetic factors are associated with IA.[4,5]

To clarify the genetic component of IA, we previously conducted genetic studies by using a multiplex IA family approach. Nonparametric linkage analysis revealed 3 loci located on 17cent, 19q13, and Xp22,[6] and parametric analysis revealed a locus on 19q13.[7] On 17cent, we found *TNFRSF13B* to be a candidate gene for IA.[8] Several other studies have revealed several loci or candidate genes in different populations.[9–19]

In 2008, Helgadottir et al[20] reported that the locus tagged by rs10757278 on chromosome 9p21 is a risk factor for IA, but not for diabetes mellitus. Bilguvar et al[21] reported a multistage, genome-wide association study of European and Japanese populations and identified 3 common single-nucleotide polymorphisms (SNPs) associated with IA on chromosomes 2q, 8q, and 9p. The aim of the present study was to investigate whether these associations could be replicated in the multiplex IA families and in sporadic Japanese IA cases.

Subjects and Methods

Study Population

Subjects from 2 groups participated. The first group consisted of subjects from previously reported families and from 3 additional families: 96 cases (male n=33; female n=63) from 31 pedigrees (Figure 1). Among the 29 previously reported pedigrees,[6] some of

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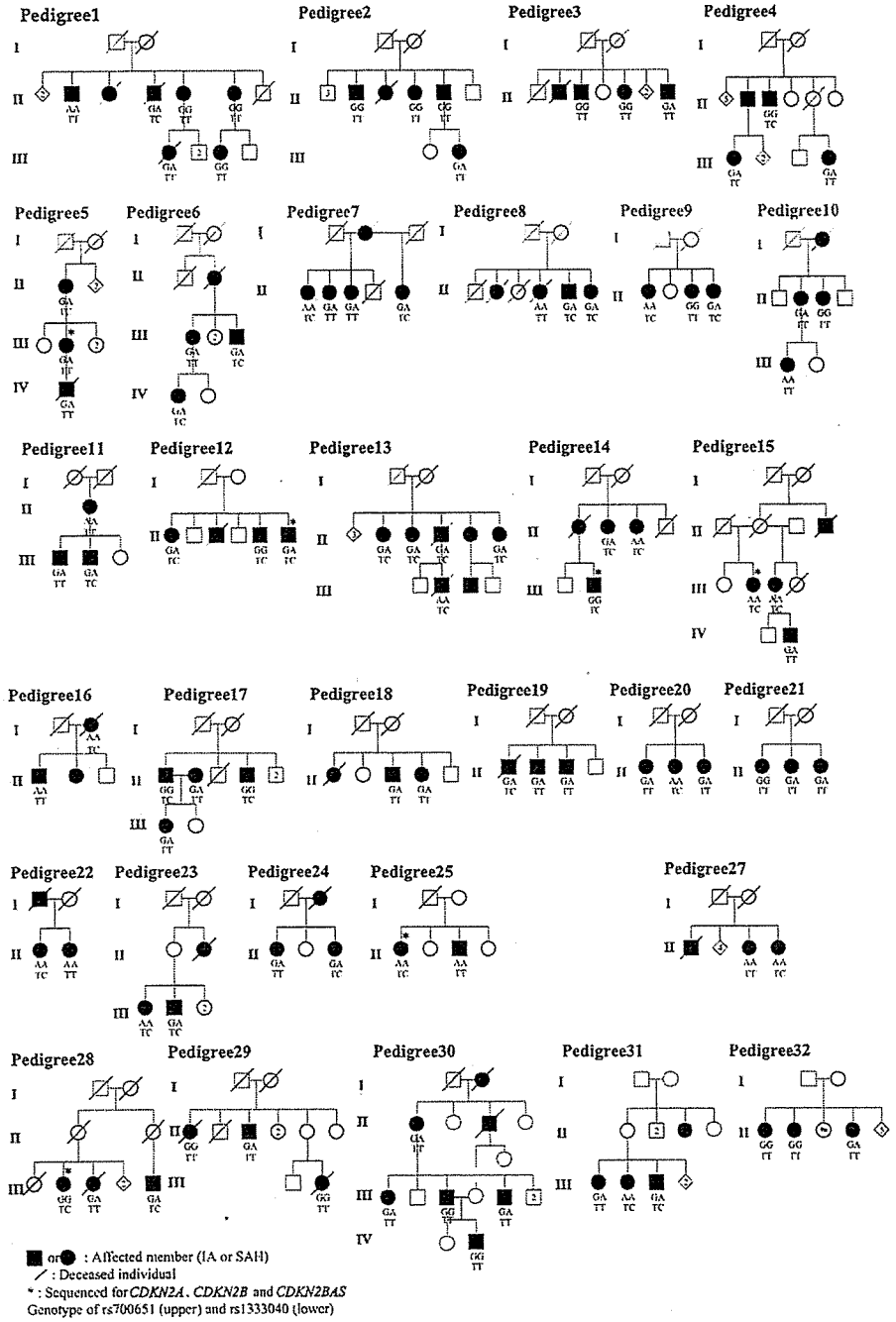

Figure 1. Pedigrees of families with IA and genotypes of individuals for rs700651 and rs1333040. Genotypes of 96 affected members are shown. Some cases have died since donating DNA samples. The identification numbers of the pedigrees are the same as previously reported.[6,8] Owing to depletion of DNA, pedigree 26 was eliminated from the figure.

the affected members could not be genotyped for rs700651, rs10958409, and rs1333040 because the DNA was exhausted by use in previous studies.

Pedigrees were recruited as previously reported.[6] In short, when cases had ≥3 living family members with IAs or ≥2 living family members with SAH, including the index cases, their families were regarded as suitable for the present study. The index cases were confirmed to have saccular aneurysms from medical records. Clinical interview and screening by magnetic resonance angiography was performed in all available relatives age 30 years or older. In subjects suspected to have IAs large enough for clinical intervention, an additional examination (digital subtraction angiography or 3-dimensional computed tomography angiography) was conducted. In addition, 46 pedigree subjects (male n=24; female n=22) who did not meet the original inclusion criteria for genetic analysis because of their young age or phenotypic uncertainty were genotyped for the current study.

The second group consisted of 419 sporadic unrelated cases (male n=142; female n=277) and 408 unrelated controls (male n=196; female n=212) collected from several collaborative hospitals in

Japan. Sporadic cases were diagnosed by digital subtraction angiography or were confirmed to have IAs during surgery. Control subjects met the following criteria: (1) confirmation of the absence of IA by digital subtraction angiography, magnetic resonance angiography, or 3-dimensional computed tomography angiography; (2) an age at examination of ≥40 years; (3) no medical history of any stroke, including IA or SAH; and (4) no family history of IA or SAH in first-degree relatives.

For all affected participants, except the 46 newly genotyped subjects, past history, lifestyle (current smoking habit and drinking habit), and comorbidity were examined from clinical records or from questionnaires conducted during interview, as previously reported.[6] For the 46 newly genotyped subjects, only their ages and relationships within pedigrees were known, whereas their IA status, comorbidities, and lifestyles were unexplored. The participation of these individuals was expected to provide greater genotype certainty in the family studies. We excluded cases or families with autoimmune diseases (including systemic lupus erythematosus, rheumatoid arthritis, and Takayasu arteritis) or known heritable diseases (including Ehlers-Danlos syndrome type IV, Marfan syndrome, neurofibromatosis type 1, and autosomal-dominant polycystic kidney disease).

This study was approved by the ethics committee of the Kyoto University institutional review board, and appropriate informed consent was obtained from all subjects.

Genotyping

We performed genotyping of 8 SNPs (rs700651, rs10958409, rs496892, rs10965215, rs10120688, rs1333040, rs7341791, and rs10757278) by using the polymerase chain reaction invader assay with TaqMan probes (Applied Biosystems TaqMan SNP genotyping assays, Foster City, Calif). The rationale for selecting the 9p SNP set, rs496892, rs1333040, and rs10757278, was the linkage disequilibrium (LD) structure of SNPs on 9p21.3. The LD block can be divided into 2 major blocks: 1 associated with vascular diseases (vascular disease block) and 1 with diabetes mellitus (diabetes block).[20] The SNPs selected are in the vascular disease block.[20–22] The physical distances between rs496892 and rs1333040 and between rs1333040 and rs10757278 are 59 and 41 kb, respectively. rs10965215, rs10120688, and rs7341791 were selected on the basis of the sequencing results of cyclin-dependent kinase inhibitor 2B antisense RNA (*CDKN2BAS*).

Linkage Analysis and Transmission Disequilibrium Test for IA Pedigrees

Two-point nonparametric logarithm of the odds scores were calculated with GENEHUNTER (version 2.1_r6)[23] for 3 SNPs (rs700651, rs10958409, and rs1333040) in the 96 affected members and in the 46 newly genotyped subjects with a disease frequency of 0.001, a phenocopy frequency of 0.02, and a penetrance of 0.70.[6] Allele frequencies of markers were set at those in controls.

To test for association of SNPs with the familial IA phenotype, we conducted the transmission disequilibrium test with TDTae software developed by Gordon et al.[24] We selected GHLO (Gordon Heath Liu Ott) error model parameters without setting the mode "inheritance."[25] In these analyses, phenotype was treated as "unknown" for the 46 newly genotyped subjects.

Association Study of SNPs With IA

An association study was performed on the 419 sporadic unrelated cases and 408 unrelated control subjects wit the SNP&Variation suite v7 (Golden Helix Inc, available at http://www.goldenhelix.com/) with or without adjustment for covariates, including sex, age, current smoking habit, and hypertension. D′ and r^2 values were also calculated for SNPs around rs1333040. Statistical power was estimated with a power calculator (available at http://pngu.mgh.harvard.edu/≈purcell/gpc/cc2.html).[26] The statistical power of the present study was 76% for α=0.05 when the frequency of the risk allele was 0.3 with a relative risk of 1.5 when D′=0.9 for a genetic marker.

Table 1. Characteristics of Familial Cases, Sporadic Cases, and Controls

Characteristic	Familial Cases	Sporadic Cases	Controls	*P*
Subjects, n, male: female	96 (33:63)	419 (142:277)	408 (196:212)	0.000086*
Cases per pedigree, mean±SD	3.10±1.13			
Age, mean±SD, y	60.5±13.6	60.0±10.7	62.0±10.1	0.025†
Cases with SAH, n (%)	60 (62.5%)	241 (57.5%)		0.37*
Subjects with hypertension, n (%)	39 (40.6%)	215 (51.3%)	162 (39.7%)	0.0024*
Subjects currently smoking, n (%)	37 (38.5%)	151 (36.0%)	161 (39.5%)	0.59*

*Comparison among familial cases, sporadic cases, and controls was made by the χ^2 test.

†Comparison was made by ANOVA.

Sequencing Candidate Genes

Three genes, *CDKN2A*, *CDKN2B*, and *CDKN2BAS*, which are in the vicinity of rs1333040, were selected for sequencing in 6 arbitrarily selected index cases from the 31 families with IA. Six index cases were selected before initiation of this study without any biases from the genotyping results. For *CDKN2A* and *CDKN2B*, we conducted direct sequencing of all coding exons, putative promoter sequences (2 kb upstream from the initiation codon), and 100 bp on either side of intron-exon boundaries. For *CDKN2BAS*, we sequenced 19 exons and 100 bp on either side of intron-exon boundaries.

For analyses, we referred to genes on the NCBI MapViewer (build 37.1, available at http://www.ncbi.nlm.nih.gov/mapview/) and to HapMap-JPT (International HapMap Project, available at http://www.hapmap.org). After polymerase chain reaction amplification and purification, samples were run on an ABI Prism 3100 Avant DNA sequencer (Applied Biosystems). Primers are summarized in supplemental Table I (available online at http://stroke.ahajournals.org). We checked sequences against the SNP database (available at http://www.ncbi.nlm.nih.gov/SNP/index.html).

Statistical Analysis

We conducted statistical analysis with SNP&Variation suite v7 software (Golden Helix Inc). Multiple comparisons were not corrected. A nominal $P<0.05$ was considered significant. Proportions were compared by the χ^2 test, and means were compared by ANOVA with SAS software (version 8.2; SAS Institute Inc, Cary, NC).

Results

Demographic Characteristics of the Study Population

Demographic data of the study population are shown in Table 1. For familial cases, an average of 3.10±1.13 (ie, mean± SD) cases (range, 2 to 7 cases) per family was included in this study (Figure 1). The proportions of female cases and of those with hypertension were different among the 3 groups ($P<0.05$), whereas the proportion of cases with a current smoking habit was not different. Hypertension was more prevalent in sporadic cases, and the proportion of females was significantly greater in the control group. No significant difference between groups was found in the proportion of cases with SAH (Table 1). To avoid confounding effects from

Table 2. Significant Associations of rs700651 and rs1333040 With IA in 31 Pedigrees, Assessed by the TDT

SNP	Position	*P* for TDT	NPL Score	*P* for NPL
rs700651 (A/G)	2q33.1	0.036	−0.212	0.571
rs10958409 (G/A)	8q11.12–12.1	0.962	−0.605	0.72
rs1333040 (C/T)	9p21.3	0.002	−0.434	0.658

TDT indicates transmission disequilibrium test; NPL, nonparametric logarithm of the odds.

known risk factors (female sex, age, and hypertension), we adjusted for these factors in the association study.

Transmission Disequilibrium Test for SNP Association With IA in Familial Cases

The transmission disequilibrium test revealed significant associations of rs700651 (P=0.036) and rs1333040 (P=0.002; Figure 1 and Table 2) with IA, whereas the association of rs10958409 with IA was not significant (P=0.962). Nonparametric logarithm of the odds scores indicated that none of the SNPs were significantly linked with IA (Table 2).

Association of SNPs With IA in Sporadic Cases

We then investigated the association of rs700651, rs10958409, rs496892, rs1333040, and rs10757278 with IA in sporadic cases. A significant association of rs1333040 with IA was shown by allelic association (P=0.02) and by the additive model (P=0.04), whereas associations of the other SNPs were not significant (Table 3). SNPs rs496892, rs1333040, and rs10757278 were not in strong LD in the study population (Figure 2). The allele frequencies of the risk allele rs1333040-T in controls and in cases were 0.64 and 0.70, respectively. These values are similar to those reported in Japanese subjects (controls:cases=0.65:0.72), but the value for cases is larger than that reported for white subjects (controls:cases=0.47:0.52 in Finland and 0.55:0.62 in the Netherlands).[21]

Sequence Analysis of *CDKN2A*, *CDKN2B*, and *CDKN2BAS*

rs1333040 is located deep in the 12th intron of *CDKN2BAS*; therefore, we searched for more substantial genomic variants than rs1333040. *CDKN2A* and *CDKN2B* are reported to be associated with cell proliferation, aging, senescence, and apoptosis,[27] and the transcriptional regulation of *CDKN2BAS* is coordinated with that of *p16/CDKN2A* and *p15/CDKN2B*.[28] Therefore, we investigated whether 6 index cases from IA families harbor mutations in these genes. Sequencing failed to show new polymorphic variants in the coding regions of *CDKN2A* and *CDKN2B*. In contrast, several polymorphisms were found in *CDKN2BAS* (Table 4). It is of interest that a common haplotype was shared by the 6 index cases, who carry the rs1333040-T allele. Because rs10965215-A, rs10120688-A, and rs7341791-G were on the same haplotype, we investigated the associations of these 3 SNPs with IA. These SNPs, however, did not show either LD with rs1333040 (Figure 2) or association with IA (Table 3), indicating a possibility that the core risk haplotype is located between introns 7 and 15 of *CDKN2BAS*. The LD block for cases did not differ from that for controls (data not shown).

Discussion

Many studies have been performed to identify IA susceptibility gene(s).[6,9–14] Some loci have been confirmed but others have not.[19] Difficulties inherent in the identification of genetic risk factors are considered to be associated with population stratification, confounding nongenetic factors, or both.[29] To overcome such difficulties, large-scale studies

Table 3. Analysis of 8 SNPs for Association With IA in a Case-Control Study

SNPs	Allele, *d*/*D**	Groups	Genotype (No. of Subjects) *dd*	*dD*	*DD*	Risk Allele Frequency	HWE, *P*†	Allelic Association† OR (95% CI)/*P*	Genotype Association Models§ Additive OR (95% CI)/*P*	Dominant OR (95% CI)/*P*	Recessive OR (95% CI)/*P*
rs700651	A/G	Case	112	208	99	0.48	0.90	1.09 (0.90–1.32)	1.14 (0.94–1.39)	1.24 (0.91–1.70)	1.14 (0.82–1.59)
2q33.1		Control	122	194	92	0.46	0.38	0.39	0.19	0.18	0.44
rs10958409	G/A	Case	236	155	28	0.25	0.71	0.86 (0.69–1.07)	0.85 (0.67–1.06)	0.78 (0.59–1.04)	0.95 (0.55–1.66)
8q11.12–12.1		Control	208	170	30	0.28	0.56	0.17	0.15	0.09	0.86
rs496892	A/G	Case	51	179	189	0.66	0.39	1.01 (0.83–1.24)	1.02 (0.83–1.26)	0.87 (0.56–1.35)	1.10 (0.83–1.46)
9p21.3		Control	44	188	176	0.66	0.55	0.90	0.84	0.54	0.51
rs10965215	G/A	Case	51	180	188	0.66	0.44	1.00 (0.81–1.22)	1.01 (0.82–1.25)	0.88 (0.57–1.37)	1.07 (0.81–1.42)
9p21.3		Control	44	186	178	0.66	0.66	0.97	0.92	0.58	0.62
rs10120688	G/A	Case	51	172	196	0.67	0.17	1.03 (0.84–1.26)	1.04 (0.85–1.28)	0.90 (0.58–1.40)	1.12 (0.85–1.49)
9p21.3		Control	45	182	181	0.67	0.94	0.78	0.70	0.65	0.42
rs1333040	C/T	Case	36	180	203	0.70	0.66	1.28 (1.04–1.57)	1.25 (1.01–1.55)	1.55 (0.98–2.46)	1.26 (0.95–1.67)
9p21.3		Control	51	187	170	0.65	0.97	0.02	0.04	0.06	0.11
rs7341791	A/G	Case	45	169	205	0.69	0.25	1.12 (0.91–1.38)	1.13 (0.92–1.39)	1.29 (0.84–2.00)	1.13 (0.86–1.50)
9p21.3		Control	52	169	187	0.67	0.16	0.27	0.24	0.25	0.38
rs10757278	A/G	Case	100	196	123	0.53	0.21	1.15 (0.95–1.39)	1.19 (0.98–1.45)	1.31 (0.95–1.80)	1.23 (0.90–1.69)
9p21.3		Control	112	192	104	0.49	0.24	0.14	0.08	0.11	0.19

*Allele *D* indicates risk alleles; *d*, wild-type alleles; and OR, odds ratio.

†*P*, HWE, 2-sided probability value from testing for deviation from Hardy-Weinberg equilibrium.

‡Without adjustment for covariates.

§With adjustment for sex, age, hypertension, and current smoking habit.

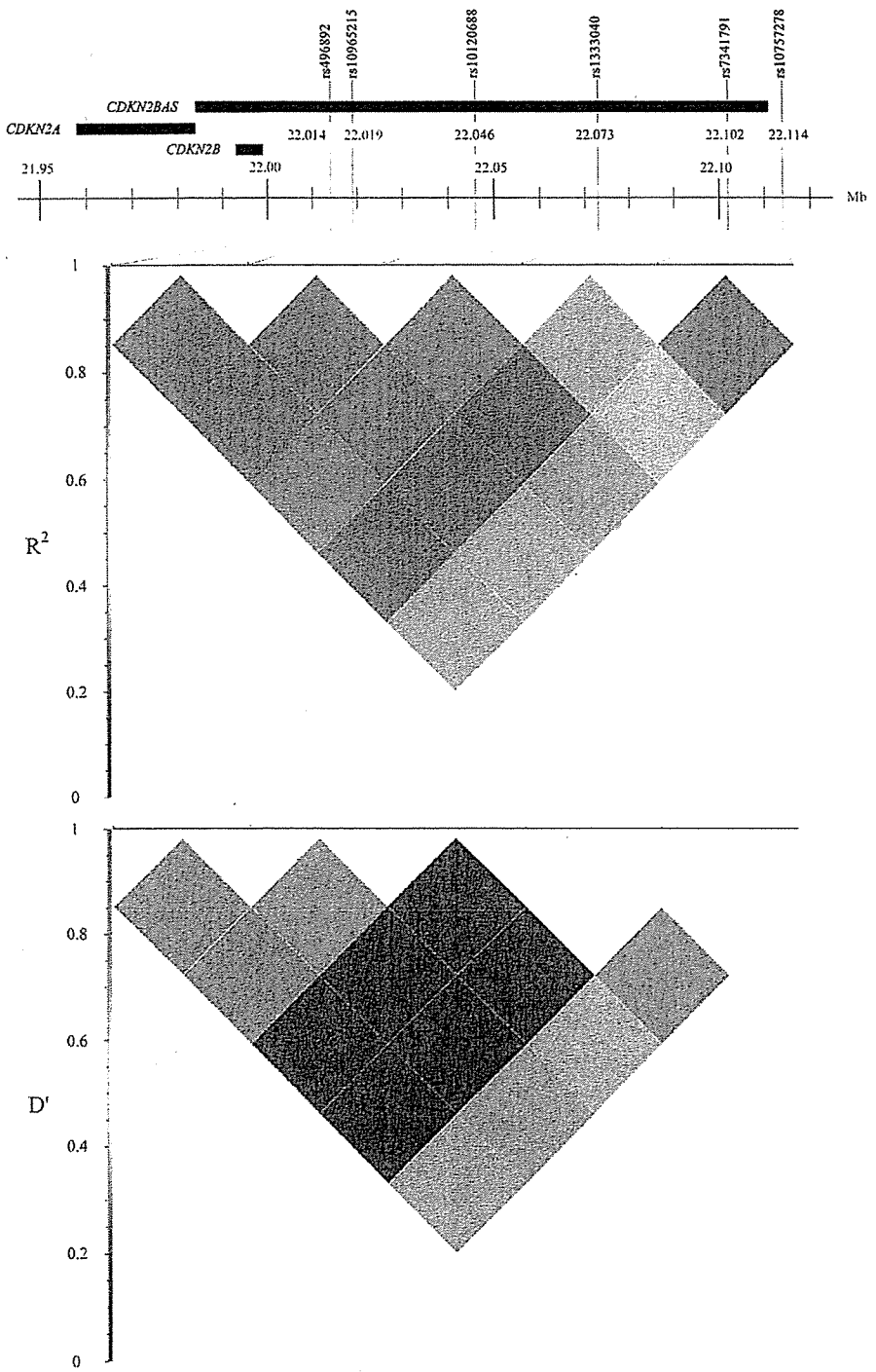

Figure 2. Six selected SNPs on 9p21.3 and LD blocks. Physical positions of 6 SNPs and related genes are illustrated, based on the NCBI database (build 37.1). LD blocks are shown by D′ and r^2 measures for a population including both cases and controls.

have been undertaken. Helgadottir et al[20] and Bilguvar et al[21] reported association studies based on a multiethnic population. Helgadottir et al[20] reported that rs10757278 (9p21) was associated with IA and that its association was independent of the diabetes block. On the other hand, Bilguvar et al[21] revealed a set of SNPs, rs700651 (2q33), rs10958409 (8q11), and rs1333040 (9p21), associated with IA.

We confirmed an association of rs1333040 with IA in 2 independent patient groups with a high or low likelihood of genetic predisposition: the multiplex IA families and sporadic cases, respectively. Such associations of rs1333040-T with IA in 2 independent populations are firmly suggestive of a substantial association with IA. To search for the gene on 9p modifying the risk of IA, we sequenced 3 genes in the vicinity

Table 4. Sequence Variants of *CDKN2A*, *CDKN2B*, and *CDKN2BAS* in Index Cases From IA Families

Gene, Nucleotide No. (Physical Position)	Position†	SNP	Description	Reference Sequence	Sequence Variants					
					Pedigree 5	Pedigree 12	Pedigree 14	Pedigree 15	Pedigree 25	Pedigree 28
CDKN2A		None								
NT_008413 17										
(21984489–21957750)										
CDKN2B		None								
NT_008413.18										
(2199931 1–21992901)										
CDKN2BAS	22019445	rs10965215*	Ex2	G	AA	AA	AA	AA	AA	AA
NT_008413.18	22036493	Unregistered	IVS4	A	AA	AA	AA	AA	AA	AC
(21984549–22111095)	22039130	rs10738605	Ex6	C	GG	GG	GG	GG	GG	GG
	22046295	rs7853090	Ex7	T	CC	CC	CC	CC	CC	CC
	22046359	rs7866783	Ex7	A	GG	GG	GG	GG	GG	GG
	22046499	rs10120688*	IVS7	G	AA	AA	AA	AA	AA	AA
	22052134	rs1011970	IVS9	G	GG	GG	GG	GG	GG	GT
	22055657	rs1333039	IVS10	G	CC	CC	GC	GC	CC	CC
	22056363	rs4977755	IVS12	T	AA	AA	AA	AA	AA	AA
	22086417	Unregistered	Ex13	T	TC	TT	TT	TT	TT	TT
	22102241	rs7341786	IVS14	A	CC	AC	AC	AC	AC	AC
	22102427	rs7341791*	IVS15	A	GG	AG	AG	AG	AG	AG
	22110371	Unregistered	Ex18	G	GG	GA	GG	GA	GG	GG
	22110490–22110491	rs67452501	IVS18 (insT)	−/−	ins/ins	−/ins	−/ins	−/−	−/ins	−/ins
	22110798–22110800	rs71949643	Ex19 (delCAT)	−/−	del/del	del/del	del/del	del/del	del/del	del/del

Ins indicates insertion del; deletion.
*SNPs used for genotyping.
†According to the NCBI MapViewer (build 37.1).

of rs1333040: *CDKN2A*, *CDKN2B*, and *CDKN2BAS*. Genetic transmission suggests that a haplotype shared by 6 index cases carries the risk allele of rs1333040-T. Three SNPs (rs10965215, rs10120688, and rs7341791), however, did not demonstrate either an association or LD with rs1333040, suggesting that a region carrying the rs1333040-T allele between introns 7 and 15 of *CDKN2BAS* may confer a bona fide risk for IA.

The 9p21 locus is associated not only with vascular diseases but also with diabetes mellitus.[20] The LD structure of this locus was investigated rigorously by Helgadottir et al[20] and was found to be composed of 2 separate LD blocks: vascular disease and diabetes blocks. The vascular disease block was tagged by SNPs, rs10757278 or rs1333040, which are in strong LD. The present study, however, failed to show a strong LD for these SNPs in the Japanese population. In the current population, IA was associated with rs1333040 but not with rs4496892 or rs10757278. More important, the current study suggests the possibility of another small LD block between introns 7 and 15 of *CDKN2BAS*, which is tagged by rs1333040.

There are several limitations of this study. The major limitations are the size and single ethnicity of the study population. Although we could not detect an association of the other 7 SNPs with IA in sporadic cases, this might have been due to insufficient statistical power because of the limited size of the study population. The limited statistical power, with a maximum sensitivity of 76%, may have failed to detect associations of other SNPs with IA, including rs10757278. In addition, we did not correct for multiple comparisons in the association studies. Another limitation was the inability to provide a biological explanation for the locus associated with IA. We could not evaluate, either directly or indirectly, any functional effect of *CDKN2BAS* with respect to IA. A major strength of the study, however, was elucidation of the fine structure of the LD block in the vicinity of rs1333040.

In conclusion, we succeeded in replicating the association of rs1333040 on 9p21 with IA in 2 independent IA patient groups and suggest that the region between introns 7 and 15 of *CDKN2BAS* may be a risk modifier. Further study is needed to elucidate the biological mechanism of this association.

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Disclosures

None.

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Effects of 0.6 mg/kg Intravenous Alteplase on Vascular and Clinical Outcomes in Middle Cerebral Artery Occlusion

Japan Alteplase Clinical Trial II (J-ACT II)

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Background and Purpose—The purpose of this study was to evaluate further the efficacy of 0.6 mg/kg intravenous alteplase on vascular and clinical outcomes in patients with middle cerebral artery occlusion in a postmarketing Phase IV trial of prospective cohort study design.

Methods—Alteplase was given intravenously at 0.6 mg/kg to patients with ischemic stroke within 3 hours of onset with MR angiography-documented middle cerebral artery occlusion. Vascular outcome was evaluated by MR angiography at 6 and 24 hours after symptom onset based on the modified Mori grade. The primary end points also included a favorable outcome (modified Rankin Scale 0 to 1 at 3 months after onset) and incidence of symptomatic intracranial hemorrhage within 36 hours after treatment. The impact of recanalization on clinical outcome was assessed by stepwise logistic regression analysis.

Results—Fifty-eight patients were enrolled. Recanalization was noted in 51.7% on 6-hour MR angiography and 69.0% on 24-hour MR angiography. A favorable clinical outcome was achieved in 46.6%. None had symptomatic intracranial hemorrhage. In logistic regression models, recanalization on either 6-hour or 24-hour MR angiography was an independent predictor for clinical outcome as well as the baseline National Institutes of Health Stroke Scale score.

Conclusions—Early recanalization of an occluded middle cerebral artery can be provoked by 0.6 mg/kg intravenous alteplase and may induce a favorable clinical outcome. The rates of recanalization and favorable outcome are comparable to that previously reported with the 0.9-mg/kg dose. (*Stroke*. **2010;41:461-465.**)

Key Words: acute ischemic stroke ■ middle cerebral artery occlusion ■ magnetic resonance angiography ■ recanalization ■ tissue plasminogen activator

Based on the Japan Alteplase Clinical Trial (J-ACT) in 2002 to 2003,[1] the Ministry of Health, Labor and Welfare of Japan approved alteplase at 0.6 mg/kg for treating acute ischemic stroke within 3 hours of symptom onset in October 2005. Although the internationally recommended dosage is 0.9 mg/kg, the 0.6-mg/kg dose had been selected according to previous tissue plasminogen activator data in Japan.[2–4] The underlying rationale has been published on the *Stroke* web site (http://stroke.ahajournals.org/cgi/content/full/37/7/1810).[1] In J-ACT, the efficacy and safety of 0.6 mg/kg intravenous alteplase for ischemic stroke were examined in a prospective cohort study and were compared with data reported for 0.9 mg/kg alteplase in North America and the European Union; the efficacy and safety profiles were compatible with those in the National Institute of Neurological Disorders and Stroke study[5] and those in a meta-analysis of data for 0.9 mg/kg. One of the conditions required by the Ministry of Health, Labor and Welfare at the time of approval was that the dosage efficacy, including potential for occluded artery recanalization, should be documented in an angiography-based study. J-ACT II is thus a prospective cohort study, in which vascular outcome, that is, recanalization of an occluded middle cerebral artery, was documented by MR angiography (MRA) as well as clinical outcome. Recanalization of occluded arteries directly reflects the pharmacological effect of thrombolytics, and early recanalization after thrombolytic therapy represents a powerful factor affecting clinical outcome.[6]

Methods

J-ACT II, a prospective, single-dose, open-label, multicenter, Phase IV trial, was performed at 15 centers in Japan between March 2007

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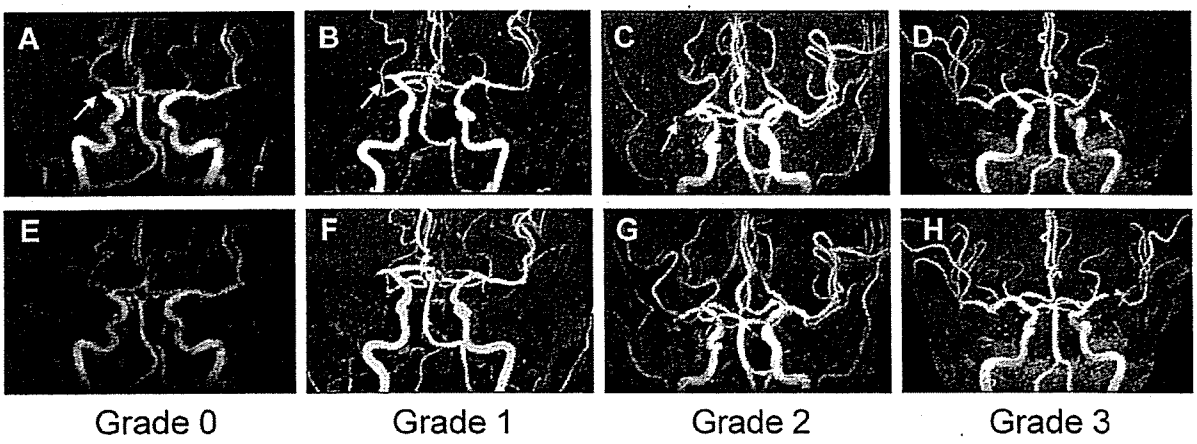

Figure. Modified Mori grades. A–D, Baseline MRA; E–H, follow-up MRA. For details of the grades, see text under "MRA Protocol." The arrow shows the occluded artery.

and July 2008. The protocol was approved by the Institutional Review Board at each center. Written informed consent was obtained from each patient or an appropriate family member before participation in this study. The patients with ischemic stroke within 3 hours of onset whose responsible arterial occlusion was identified in the middle cerebral artery (M1 or M2 segment) by MRA were given 0.6 mg/kg intravenous alteplase with 10% being administered as a bolus followed by continuous infusion of the remainder over 1 hour. Exclusion criteria were adopted from the National Institute of Neurological Disorders and Stroke rtPA Stroke Study[5] and J-ACT.[1] Also excluded were patients whose National Institutes of Health Stroke Scale (NIHSS) score was ≥23, those contraindicated for MRI, those whose MRA demonstrated arterial occlusions other than of the middle cerebral artery, or whose Alberta Stroke Program Early Computed Tomography Score (ASPECTS) was ≤6. Only CT and MRA were considered for subject selection, although diffusion-weighted images were also obtained to investigate their role in selecting patients (data to be reported elsewhere).

MRA Protocol

Before the study, the MRI conditions were standardized to unify the image quality among all participating sites. For MRI and MRA, a 1.5-T echoplanar imaging-equipped scanner was used. Three-dimensional time-of-flight MRA was performed under the following conditions: axial images parallel to the anterior commissure–posterior commissure plane; scanning range from the pontomedullary junction to the corpus callosum; slice thickness 1 to 1.5 mm; and field of view 200 to 240 mm. MRA images were processed by maximum intensity projection to create images of the axial projection and in rotation about the vertical axis (RL rotation, 15° to 18°). MRA was repeated at baseline, 6 hours, and 24 hours after symptom onset. The time allowance for 6-hour MRA was between the end of alteplase infusion and 8 hours from symptom onset and that for 24-hour MRA was between 24 and 36 hours after symptom onset. Arterial occlusion was assessed by 2 reviewers, one expert neurologist and one expert neuroradiologist (the image reading panel) blinded to information except the affected side. Recanalization was evaluated according to the modified Mori grade: Grade 0, no reperfusion; Grade 1, movement of thrombus not associated with any flow improvement; Grade 2, partial (branch) recanalization in <50% of the branches in the occluded arterial territory; and Grade 3, nearly complete recanalization with reperfusion in ≥50% of the branches in the occluded-arterial territory (Figure). Modifications were made to apply the original scheme,[2] which was developed for conventional angiography, to MRA, because distal arterial branches are not visible on MRA. The recanalization rate was estimated by regarding Grades 2 and 3 as valid recanalization corresponding to Thrombolysis in Myocardial Infarction Grades 2 and 3.

Clinical Evaluations

As a primary outcome, the functional outcome after 3 months was assessed by the modified Rankin Scale (mRS). Symptomatic intracranial hemorrhage was designated as CT evidence of intracranial hemorrhage accompanied by apparent neurological deterioration defined as conditions that could be documented objectively or were increased by ≥4 points from the latest NIHSS score. CT images obtained at 24 to 36 hours were assessed by the image reading panel. According to the European Cooperative Acute Stroke Study CT criteria, the panel classified hemorrhagic transformation as none, hemorrhagic infarction (HI-1 and HI-2), or parenchymal hematoma (PH-1 and PH-2).

End Points

The primary end points were modified Mori Grade 2 and 3 recanalization on 6-hour MRA and 24-hour MRA and a favorable outcome of mRS 0 to 1 at 3 months. The safety primary end point was symptomatic intracranial hemorrhage within 36 hours. If data were missing at any follow-up time point, data were imputed using the "last observation carried forward."

To test the hypothesis, we used a similar strategy to the one-arm trial, J-ACT[1]: the incidences of the primary end points were compared with the results of a meta-analysis of published data on thrombolysis. First, we searched MEDLINE and Current Contents as of March 2006 using the following key words: (acute stroke OR ischemic stroke) AND tPA AND angiography. Publications incorporating information concerning the present primary end points were selected to determine the target reference values. Based on the 5 publications selected,[2,3,7–9] we determined a target value for the recanalization rate on 6-hour MRA; the weighted average recanalization rate was 45.1% in 113 patients. The 90% CI of the recanalization rate in 50 patients (the target patient number for this study) was estimated to be 33.5% to 56.8% (normal approximation without sequential correction). In the present study, the treatment aim was thus for a recanalization rate of not <33.5%, the lower limit of the 90% CI. Similarly, we determined a target value for the recanalization rate on 24-hour MRA of not <57.7% based on one publication.[10]

Second, we repeated the database survey with a different search strategy: (acute stroke OR ischemic stroke) AND middle cerebral artery AND (tissue plasminogen activator OR urokinase OR prourokinase). Based on the 2 publications found in the literature search[11,12] and unpublished data from the Middle Cerebral Artery Embolism Local Fibrinolytic Intervention Trial (MELT-J), which was published during this study,[13] we estimated the weighted mean proportion of patients with a favorable outcome at 3 months to be 33.6% and the 90% CI in 50 patients to be 22.6% to 44.6%. From data in 3 publications[12,14,15] and MELT-J,[13] we estimated the

Table 1. Demographics and Baseline Characteristics of Patients (n=58)

Age, years	70.3 (11.5)
Sex, females	23 (39.7%)
Body weight, kg	62.1 (11.7)
Baseline NIHSS	12 (5–22)
Stroke subtype	
Cardioembolic	49 (84.5%)
Atherothrombotic	5 (8.6%)
Other/not differentiated	4 (6.9%)
M1 occlusion	41 (70.7%)
Systolic blood pressure, mm Hg	148.5 (16.2)
Diastolic blood pressure, mm Hg	81.2 (12.1)
Blood glucose, mg/dL	132.9 (46.2)
Time elapsed, hours	
Onset to treatment	2.2 (0.4)
Onset to 6-hour MRA	5.9 (1.4)
End of tPA infusion to 6-hour MRA	2.7 (1.3)
Onset to 24-hour MRA	27.1 (2.7)
End of tPA infusion to 24-hour MRA	23.9 (2.7)

Data show the mean (SD), median (range), or no. (%).
tPA indicates tissue plasminogen activator.

weighted mean incidence of symptomatic intracranial hemorrhage to be 8.2% and the 90% CI in 50 patients to be 1.8% to 14.6% for use as reference values.

Statistical Analysis

The effect of recanalization on clinical outcome was assessed by comparing the proportion of a favorable outcome at 3 months between patients with and without recanalization using Fisher exact test, which was also expressed as the ORs and 95% CI. To examine the effects of baseline characteristics and recanalization on clinical outcome, disease-related factors, including time from onset, hypertension, diabetes mellitus, baseline NIHSS, occluded site (M1 or M2), and ASPECTS, and recanalization on either 6-hour MRA or 24-hour MRA were included in a stepwise regression analysis, in which age and sex were forcibly entered into the model to adjust for their possible confounding effects. To assess the possible interaction of recanalization with severity of disease/ischemia, interaction terms between recanalization and NIHSS, ASPECTS, or occlusion site were entered into the model. Furthermore, to examine the effect of delayed recanalization (ie, arterial occlusion unchanged on 6-hour MRA but recanalized on 24-hour MRA), a similar analysis was repeated, in which both delayed recanalization and early recanalization on 6-hour MRA were entered into the model. Significance was set at $P<0.05$ in all final models. The OR and 95% CI were also determined. SAS 9.1.3 was used for the statistical analyses.

Results

Fifty-eight patients were enrolled in this study and were included in the full analysis set both for primary safety and for primary efficacy. One patient had no occluded artery on baseline MRA according to the image reading panel and was excluded from further analysis. Table 1 summarizes the patients' characteristics.

The recanalization rate on 6-hour MRA was 51.7% (Table 2). The recanalization rate did not differ significantly between M1 and M2 occlusions (48.8% versus 62.5%, respectively; $P=0.391$). In all except 2 patients who were withdrawn or had an obstacle for MRI, 24-hour MRA was available. The recanalization rate on 24-hour MRA was 69.0% (Table 2). Delayed recanalization was noted in 10 patients (17.5%). No patient had recanalization on 6-hour MRA that subsequently disappeared on 24-hour MRA.

Table 2. Vascular Conditions and Recanalization After Thrombolysis

Modified Mori Grade	0	1	2	3	Recanalization Rate (95% CI)*
6-hour MRA (n=58)					51.7 (38.9–64.6)
n	21†	7	3	27	
Percent	36.2	12.1	5.2	46.6	
24-hour MRA (n=58)					69.0 (57.1–80.9)
n	12†	6	4	36‡	
Percent	20.7	10.3	6.9	62.1	

*Valid recanalization (Mori Grade 2 or 3) and 95% CI.
†Including one patient whom the image reading panel judged as having no occlusion on baseline MRA.
‡Including 2 patients in whom data were imputed using the "last observation carried forward" for missing 24-hour MRA.

Three-month clinical outcomes were unavailable in 2 patients; one withdrew consent and the other was discharged earlier with an mRS of 4. Both were categorized as having an "unfavorable outcome." The proportion of a favorable outcome at 3 months was 46.6% (95% CI, 33.7% to 59.4%). Death within 3 months after onset occurred in one patient (1.7%), who died of septic shock at 50 days after entry. An alteplase-related serious adverse event occurred in one patient, who had an ischemic stroke on the side opposite to the original stroke 12 hours after alteplase infusion.

The proportion of a favorable outcome was significantly higher in patients with recanalization than in those without recanalization on either 6-hour or 24-hour MRA (Table 3). In a logistic regression model with 6-hour MRA entered as an independent variable, recanalization (OR, 6.030; 95% CI, 1.730 to 21.011) and baseline NIHSS (OR, 0.841; 95% CI, 0.719 to 0.983) emerged as independent predictors of a favorable outcome. In another model with 24-hour MRA entered, recanalization (OR, 21.231; 95% CI, 3.318 to 135.859) and baseline NIHSS (OR, 0.796; 95% CI, 0.672 to 0.943) were also independent predictors of a favorable outcome. The model with delayed recanalization revealed 6-hour recanalization (OR, 23.036; 95% CI, 3.474 to

Table 3. Relationship Between Vascular Outcome and Clinical Outcome at 3 Months

Time	Favorable (mRS 0–1)	Unfavorable (mRS ≥2)	OR [95% CI] Probability
6-hour MRA			
Recanalized	20 (66.7%)	10 (33.3%)	5.714 [1.814–18.004]
Not recanalized	7 (25.9%)	20 (74.1%)	$P=0.003$
24-hour MRA			
Recanalized	25 (62.5%)	15 (37.5%)	12.500 [2.503–62.428]
Not recanalized	2 (11.8%)	15 (88.2%)	$P<0.001$

152.753), delayed recanalization (OR, 15.949; 95% CI, 1.710 to 148.762), and baseline NIHSS (OR, 0.801; 95% CI, 0.675 to 0.951) as independent predictors of a favorable outcome.

No patient had symptomatic intracranial hemorrhage within 36 hours. Asymptomatic intracranial hemorrhage was present in 19.0% of patients (11 of 58) on CTs at 24 to 36 hours, but no patient had parenchymal hematoma 2.

Discussion

This is the first prospective multicenter clinical trial to evaluate recanalization of occluded arteries by MRA shortly after tissue plasminogen activator administration and at 24 hours. The recanalization rates immediately (2.7 hours on average) after treatment and at 24 hours (23.9 hours on average) after treatment were 51.7% and 69.0%, respectively, exceeding the predetermined thresholds. A systematic review in May 2009 revealed that the weighted average of the recanalization rate in the placebo arm of randomized controlled trials of thrombolysis examined by conventional angiography or MRA was 19.8% up to 8 hours after onset.[2,3,12,16] The recanalization rate in the present study was thus considered likely to be much higher than the rate of spontaneous recanalization.

Concerning clinical outcomes, the proportion of a favorable outcome at 3 months (46.6%) fairly well exceeded the predetermined threshold. The systematic review in May 2009 revealed that the weighted average of the proportion of a favorable outcome (mRS 0 or 1) for patients with middle cerebral artery occlusion in the placebo arm of randomized controlled trials of thrombolysis was 22.3%.[11–13,16] The proportion of a favorable outcome in the present study was considered likely to be much higher than that in the natural course of patients with middle cerebral artery occlusion.

The present findings indicated that 0.6 mg/kg intravenous alteplase is, as expected, effective in terms of vascular and clinical outcomes. The most critical limitations of this study arise from the lack of a control group, a postmarketing clinical trial of open-label design, and comparison of results with published data, which could generate various biases. Although primary vascular outcome was assessed centrally by raters independent from the participating sites, rater prejudice cannot be excluded. Nevertheless, the MRA imaging conditions were standardized among all participating sites, and 2 expert raters reviewed the images blinded to the clinical information, probably ensuring quality of image acquisition and evaluation.

Concerning safety, we did not encounter symptomatic intracranial hemorrhage in this trial, which was much better than expected. However, this could reflect the small sample size used. In the Phase III clinical study (J-ACT),[1] symptomatic intracranial hemorrhage occurred in 5.8% of patients, whose arterial occlusions were not documented. Asymptomatic intracranial hemorrhage was noted in 19% of the present subjects, which was comparable to that in the previous trial (17%).[1]

Recanalization immediately after any form of thrombolysis has repeatedly been indicated to predict clinical outcome.[2–4,7,9] A recent systematic review of cerebral artery recanalization has confirmed a strong correlation between recanalization and clinical outcome in acute ischemic stroke.[6] Several investigations have also suggested that the baseline severity of symptoms as measured by NIHSS represents an independent predictor for clinical outcome in patients treated with intravenous alteplase.[11,17,18] Similar to previous thrombolysis studies, the present results demonstrated a strong relationship between vascular outcome and functional outcome as well as baseline stroke severity. Recanalization on either 6-hour or 24-hour MRA was an independent predictor for a favorable clinical outcome. Our data indicated that recanalization on 24-hour MRA was a much stronger predictor of clinical outcome than that on 6-hour MRA. These findings should be interpreted cautiously; they do not necessarily imply that delayed recanalization is far more effective than early recanalization, because recanalization on 24-hour MRA is a cumulative result. Nevertheless, delayed recanalization (recanalization occurring between 6 and 24 hours after treatment) was also a modest but independent predictor for a favorable outcome. The prognostic value of the 24-hour cumulative recanalization is supported by a transcranial Doppler study.[19] Delayed as well as early recanalization may thus have a favorable impact on clinical outcome.

In conclusion, early recanalization of an occluded middle cerebral artery can be provoked by 0.6 mg/kg intravenous alteplase and may induce a favorable clinical outcome. The rates of recanalization and a favorable outcome are comparable to that previously reported with the 0.9-mg/kg dose.

Appendix

Steering Committee

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Stroke

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The Relationship Between Waist Circumference and the Risk of Stroke and Myocardial Infarction in a Japanese Urban Cohort: The Suita Study

Yoko Furukawa, Yoshihiro Kokubo, Tomonori Okamura, Makoto Watanabe, Aya Higashiyama, Yuu Ono, Katsuyuki Kawanishi, Akira Okayama and Chigusa Date

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Research Letters

The Relationship Between Waist Circumference and the Risk of Stroke and Myocardial Infarction in a Japanese Urban Cohort

The Suita Study

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Background and Purpose—Body mass index is most commonly used as the obesity index. Recently, waist circumference (WC) has been shown to be associated with the risk of cardiovascular disease (CVD). However, no studies have observed an association between WC and CVD in Japan. We examined the relationships of WC and body mass index with CVD in a Japanese urban population.

Methods—We studied 5474 Japanese individuals (aged 30 to 79 years without CVD at baseline) who completed a baseline survey and received follow-up through December 2005. WC was measured at the umbilical level of participants in the standing position to the nearest 1 cm. The Cox proportional hazard ratios for CVD according to the quartiles of WC were calculated after adjustment for age, smoking, and drinking status.

Results—During a mean follow-up of 11.7 years, 207 strokes and 133 myocardial infarctions were documented. In women, compared with the lowest quartile (WC <70 cm), the hazard ratio (95% CIs) after adjusting for age, smoking, and drinking in the highest quartile (WC ≥84 cm) were 1.85 (1.03 to 3.31) for CVD and 2.64 (1.16 to 6.03) for stroke. However, no such relationships of WC with CVD or stroke risk were observed in men. After further adjustment of hypertension, diabetes, and hypercholesterolemia, all of the mentioned relationships were not statistically significant. No associations of body mass index with CVD or strokes were observed.

Conclusions—WC may be a better predictor for CVD or stroke in Japanese women. (***Stroke*. 2010;41:550-553.)**

Key Words: abdominal obesity ■ cardiovascular disease ■ epidemiology ■ prospective studies ■ stroke ■ waist circumference

The increasing prevalence of obesity worldwide has led to concern about the impact of obesity on the risk of cardiovascular disease (CVD).[1] Body mass index is most commonly used as the measurement of obesity. Recently, abdominal obesity measured by waist circumference (WC) has been shown to be associated with the risk of CVD.[2,3] However, no cohort study on the relationship between WC and the risk of CVD has been performed in Japan. We therefore examined the relationship of WC and body mass index with the incidence of CVD and stroke in a Japanese urban population.

Methods

The Suita Study starting from September 1989, a random sampling of Japanese urban residents, has been described previously (response rate: 53%).[4–6] Participants (n=5474) who attended the baseline examination without a history of CVD were followed up. The details of the methods and confirmation of stroke and myocardial infarction in the Suita Study have been described elsewhere.[4–6] The follow-up was continued until one of the following end points, whichever came first: date of the first myocardial infarction or stroke event, date of death, date of leaving Suita, or December 31, 2005. This study was approved by the Institutional Review Board of the National Cardiovascular Center.

Analyses of variance and χ^2 tests were used to compare mean values and frequencies. The Cox proportional hazard ratios and 95% CIs for CVD were calculated according to the quartiles of WC after adjusting for age, smoking, and drinking status. All statistical analyses were performed with SPSS (Version 13.0J; SPSS Japan Inc, Tokyo, Japan).

Results

During a mean follow-up of 11.7 years, we documented 207 strokes and 133 myocardial infarctions. As shown in Table 1, both men and women with higher WC were older; had a

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Table 1. Baseline Characteristics of Subjects According to Quartile of WC by 2560 Men and 2914 Women Aged 30 to 79: The Suita Study, 1989–1994, Japan

	WC				
	Q1 (Low)	Q2	Q3	Q4 (High)	*P*
Women					
No. of subjects	702	679	739	794	
Waist circumference, cm*	54–69	70–75	76–83	84–121	<0.001
Age, years*	49±13	52±13	55±12	60±11	<0.001
Body mass index, kg/m^2*	19.6±1.9	21.1±2.0	22.5±2.2	25.3±3.2	<0.001
Hypertension, %†	14	20	27	46	<0.001
Diabetes, %†	1	2	3	7	<0.001
Hypercholesterolemia, %†	32	36	45	54	<0.001
Smoking status (current/quit/never), %†	14/3/83	12/3/85	12/3/85	11/5/84	0.092
Drinking status (current/quit/never), %†	34/1/64	34/2/63	32/1/67	31/1/67	0.512
Men					
No. of subjects	564	711	627	658	
Waist circumference, cm*	57–76	77–82	83–87	88–124	<0.001
Age, years*	56±14	54±13	56±13	57±12	0.003
Body mass index, kg/m^2*	19.7±1.8	21.9±1.6	23.5±1.6	25.9±2.3	<0.001
Hypertension, %†	24	29	35	46	<0.001
Diabetes, %†	4	6	5	9	0.013
Hypercholesterolemia, %†	19	27	33	34	<0.001
Smoking status (current/quit/never), %†	56/27/17	55/24/20	45/37/18	44/35/21	<0.001
Drinking status (current/quit/never), %†	71/4/25	76/4/28	77/4/19	76/4/20	0.287

Data indicate frequencies or means±SDs.

*Analysis of variance was performed.

†χ^2 test was performed.

Q indicates quartile; Hypertension, systolic blood pressure/diastolic blood pressure ≥140/90 mm Hg or current use of antihypertensive medications; diabetes, fasting plasma glucose levels ≥7.0 mmol/L or nonfasting plasma glucose levels ≥11.1 mmol/L or current use of antidiabetic medications; hypercholesterolemia, total serum cholesterol levels ≥5.7 mmol/L or current use of antihyperlipidemic medications.

higher prevalence of hypertension, diabetes, and hypercholesterolemia; and had a lower prevalence of current smokers. The correlations between body mass index and WC were 0.84 in men and 0.75 in women.

The highest quartile of WC was associated with a significant increase in the risk of CVD and stroke compared with the lowest quartile in women but not in men (Table 2). Moreover, in women, the association between WC and CVD disappeared after further adjustment for hypertension, diabetes, and hypercholesterolemia. These associations were not also statistically significant even after removing subjects with any of mentioned 3 risk factors (data not shown). When we sequentially changed the cutoff values of WC, an increased risk of CVD was observed in women with WC ≥80 cm (Figure). No associations of body mass index with CVD or strokes were observed (data not shown).

Discussion

In this cohort study, abdominal obesity (WC ≥80 cm) was positively associated with CVD in women, which is the first study of the relationships in Japan. In the 2 large cohort studies in the Western population,[2,3] WC was positively associated with the risk of CVD. However, the current study only observed the positive relationships of WC with CVD and stroke in women. The reasons for the sex difference are unclear but may involve differences in race, lifestyle background, severity of obesity, or prevalence of risk factors in nonobese subjects.

Abdominal obesity, strongly correlated with WC, has been associated with insulin resistance,[7] diabetes,[8] lipid abnormalities,[9] and blood pressure elevation.[10] The association between WC and CVD disappeared after further adjustment for cardiovascular risk factors or removing subjects with diabetes, hypertension, or hypercholesterolemia. Therefore, WC might be likely on the causal pathway leading to the more proximal risk factors for CVD and contributes to risk through those factors. Abdominal obesity without other cardiovascular risk factors does not predict the risk of CVD. However, it should be careful that increasing abdominal obesity might lead to those factors.

Our study has the following limitations: regression dilution bias, small sample size for subgroup analysis, lifestyle background, and measurement of WC at the umbilical level rather than at the midpoint between the

Table 2. Age- and Multivariable-Adjusted Hazard Ratios (95% CIs) for CVD According to WC Quartile by Sex

	Q1 (Low)	Q2	Q3	Q4 (High)	*P* for Trend
Women					
Waist circumference, cm	54–69	70–75	76–83	84–121	
Person-years	8686	8334	8880	9008	
Cardiovascular disease					
No. of cases	15	23	35	63	
Age-adjusted	1	1.27 (0.66–2.44)	1.50 (0.81–2.76)	1.84 (1.04–3.27)	0.02
Multivariable-adjusted	1	1.30 (0.67–2.54)	1.57 (0.84–2.91)	1.85 (1.03–3.31)	0.02
Stroke					
No. of cases	7	15	27	42	
Age-adjusted	1	1.82 (0.74–4.49)	2.55 (1.10–5.92)	2.80 (1.24–6.33)	0.01
Multivariable-adjusted	1	1.78 (0.71–4.46)	2.57 (1.10–6.00)	2.64 (1.16–6.03)	0.01
Myocardial infarction					
No. of cases	8	8	8	21	
Age-adjusted	1	0.83 (0.31–2.24)	0.64 (0.24–1.73)	1.12 (0.49–2.55)	0.67
Multivariable-adjusted	1	0.91 (0.33–2.49)	0.72 (0.26–1.96)	1.26 (0.54–2.97)	0.50
Men					
Waist circumference, cm	57–76	77–82	83–87	88–124	
Person-years	6443	8185	7304	7183	
Cardiovascular disease					
No. of cases	43	53	45	63	
Age-adjusted	1	1.13 (0.75–1.69)	0.91 (0.60–1.39)	1.25 (0.85–1.85)	0.41
Multivariable-adjusted	1	1.13 (0.75–1.69)	0.92 (0.60–1.40)	1.33 (0.89–1.97)	0.28
Stroke					
No. of cases	22	28	32	34	
Age-adjusted	1	1.15 (0.65–2.01)	1.22 (0.71–2.11)	1.31 (0.77–2.25)	0.31
Multivariable-adjusted	1	1.14 (0.65–2.00)	1.25 (0.72–2.16)	1.40 (0.82–2.41)	0.20
Myocardial infarction					
No. of cases	21	25	13	29	
Age-adjusted	1	1.09 (0.61–1.95)	0.55 (0.27–1.10)	1.20 (0.68–2.10)	0.92
Multivariable-adjusted	1	1.10 (0.61–1.97)	0.55 (0.27–1.10)	1.29 (0.72–2.29)	0.79

Multivariable adjustments were performed for age, smoking, and drinking status.
Q indicates quartile.

lower rib and the iliac crest. However, the correlation coefficients between values measured by using the both methods for measurement of WC were reported to be high.[11]

In conclusion, our findings suggested that WC was associated with an increased risk of CVD and stroke in Japanese women. From a public health perspective, measurement of WC could be a useful tool for use in preliminary screening for high risk of CVD.

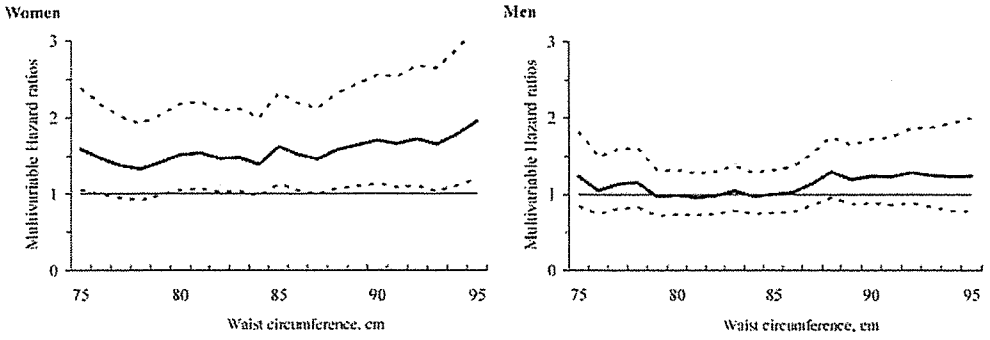

Figure. The risk for CVD through sequential changes in WC in men and women. Solid line indicates hazard ratios for CVD according to different cutoff values of WC. Dotted lines indicate 95% CIs for each WC.

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Disclosures

None.

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Left Atrial Volume Combined With Atrial Pump Function Identifies Hypertensive Patients With a History of Paroxysmal Atrial Fibrillation

Norihisa Toh, Hideaki Kanzaki, Satoshi Nakatani, Takahiro Ohara, Jiyoong Kim, Kengo F. Kusano, Kazuhiko Hashimura, Tohru Ohe, Hiroshi Ito and Masafumi Kitakaze

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7-17

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Abstract—Identifying patients at high risk for the occurrence of atrial fibrillation is one means by which subsequent thromboembolic complications may be prevented. Left atrial enlargement is associated with progression of atrial remodeling, which is a substrate for atrial fibrillation, but impaired atrial pump function is also another aspect of the remodeling. Our objective was to differentiate patients with a history of paroxysmal atrial fibrillation using echocardiography. We studied 280 hypertensive patients (age: 66±7 years; left ventricular ejection fraction: 65±8%), including 140 consecutive patients with paroxysmal atrial fibrillation and 140 age- and sex-matched control subjects. Left atrial volume was measured using the modified Simpson method at both left ventricular end systole and preatrial contraction and was indexed to body surface area. Peak late-diastolic mitral annular velocity was measured during atrial contraction using pulsed tissue Doppler imaging as an atrial pump function. Left atrial volume index measured at left ventricular end systole had a 74% diagnostic accuracy and a 71% positive predictive value for identifying patients with paroxysmal atrial fibrillation; these values for the ratio of left atrial volume index at left ventricular end systole to the peak late-diastolic mitral annular velocity were 82% and 81%, respectively, and those for the ratio of left atrial volume index at preatrial contraction to the peak late-diastolic mitral annular velocity were 86% and 90%, respectively. In conclusion, left atrial size combined with atrial pump function enabled a more accurate diagnosis of a history of paroxysmal atrial fibrillation than conventional parameters. (***Hypertension.* 2010;55:1150-1156.**)

Key Words: hypertension ■ echocardiography ■ atrial fibrillation ■ left atrium ■ remodeling

Atrial fibrillation (AF) is not generally life threatening but is considered to be the most common cause of ischemic stroke, which often yields serious complications because of acute occlusion of intracranial arteries without collateral circulation. The incidence of stroke is increased by ≈5-fold in the presence of nonvalvular AF.[1–5] Moreover, recent studies have demonstrated that stroke risk is no less in patients with paroxysmal AF (PAF) than in those with persistent AF.[6] Therefore, it is crucial to identify patients who have PAF in order to prevent subsequent thromboembolic complications, especially in patients with hypertension, which is a major etiologic factor associated with both AF and stroke.[7–9]

Left atrial (LA) enlargement associated with the progression of atrial structural remodeling plays a key role in the initiation and maintenance of AF.[10,11] The most recent recommendations for echocardiographic chamber quantification indicate that LA volume (LAV) provides an accurate measurement of asymmetrical remodeling of the LA chamber.[12] LAV is increased in patients with PAF[13] and is also an important predictor of cardiovascular outcome, including the occurrence of AF,[14] supporting the concept that LAV is a hallmark of atrial remodeling.

In patients with PAF, Doppler transmitral flow velocity and tissue Doppler imaging (TDI)–derived mitral annulus velocity during atrial contraction, which are considered to reflect atrial pump function,[15–20] have been reported to be decreased compared with those in control subjects.[13,16,21] According to the Frank-Starling law, atrial pump function is also enhanced with an increase in LAV; however, excessive LA enlargement leads to atrial dysfunction.[22,23] Accordingly, we hypothesized that adding information on atrial pump function may provide a better marker of atrial remodeling.

In the present study, we aimed to differentiate patients with a history of PAF among those with hypertension more accurately by means of LAV combined with atrial pump function.

Methods

Study Population

Patients referred to our laboratory were classified into the PAF group if they met the following criteria: (1) ≥1 episode of self-terminating

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PAF documented by a 12-lead ECG, 24-hour Holter monitoring, or continuous monitoring during hospitalization without taking any antiarrhythmic drugs and having been free from arrhythmic episodes for >1 week before undergoing echocardiography; (2) hypertension (systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure ≥90 mm Hg or treatment for hypertension); (3) less than moderate mitral regurgitation; (4) sinus rhythm during echocardiography; (5) no medical history of other arrhythmias (including persistent AF), valvular heart disease (including mitral annular calcification), heart failure, ischemic heart disease, cardiomyopathy, cardiac surgery, thyroid disease, or pulmonary disease; and (6) age between 40 and 80 years.

Control subjects were recruited from a clinical health examination in Arita, Japan. All of the attendees underwent a formal medical history interview, ECG, and physical and laboratory examinations. Blood pressure was measured at each of ≥2 visits to the office, and the average of ≥2 seated blood pressures was used according to established recommendations.[24] Of the attendees who also underwent echocardiography, age- and sex-matched subjects who met criteria 2 through 6 were classified as the control group. The study was approved by the institutional review board, and the study was conducted in accordance with the ethical principles of the Declaration of Helsinki. All of the subjects provided informed consent.

Echocardiography

All of the echocardiographic studies were performed with either a Vivid 7 Dimension (GE Healthcare) or Aplio XV (Toshiba Medical Systems) ultrasound system. Cardiac chamber size, left ventricular (LV) ejection fraction (LVEF), LV mass, and LA dimension were evaluated according to the recommendations of the American Society of Echocardiography.[12] LAV was measured using the biplane modified Simpson's method at the ventricular end-systolic frame just before the mitral valve opening from apical 4- and 2-chamber views. Strictly speaking, however, LA does not contract from the size of LAV at ventricular end systole. Thus, preatrial contraction LAV (LAV_{preA}) was also obtained from the last frame just before mitral valve reopening. Active LA emptying fraction (active LAEF) was calculated by the following formula: $(LAV_{preA}-LAV_{min})/LAV_{preA}\times100\%$, where LAV_{min} is the minimal LAV at atrial end systole. All of the LAVs were indexed to body surface area as LAVi, $LAVi_{preA}$, and $LAVi_{min}$, respectively. LV mass was also indexed to body surface area (LV mass index). LV hypertrophy was defined as LV mass index >104 g/m^2 in women and >116 g/m^2 in men.[25]

The sample volume of pulsed-wave Doppler imaging was placed at the tip level of the mitral leaflets in the apical 4-chamber view. Then the peak mitral inflow early diastolic and atrial filling (E and A) velocities and the E-wave deceleration time were obtained. The sample volume of the pulsed TDI was placed at the septal and lateral margins of the mitral annulus. Peak early and late-diastolic mitral annular velocities were measured, and then the average values of septal and lateral velocities were used as Ea and Aa, respectively. E/Ea was calculated as a surrogate for the LV filling pressure.[26]

Statistical Analysis

Data are expressed as mean±SD. An ANOVA was performed to test for statistically significant differences between 2 unpaired mean values, and categorical data and percentage frequencies were analyzed by the χ^2 test. Correlations were determined with Pearson product moment correlation analysis. Receiver operating characteristic (ROC) curves were constructed to determine the optimal sensitivity and specificity. The area under the curve (AUC) was calculated to assess the overall performance of various variables for the detection of PAF. Univariate and multivariate logistic regression analyses were performed to characterize diagnostic factors of a history of PAF. Variables considered included age, sex, body weight, LV hypertrophy, E/Ea, and diabetes mellitus. A scatter diagram was used to illustrate the relationship between LA size and LA pump function in each patient. A straight line was drawn passing through the origin to discriminate the best between the PAF and control groups, and another line was drawn to establish the boundary above which spots belonging to the PAF group existed. The slope indicates the ratio of LA size to LA pump function: the former corresponds to the optimal cutoff value from the ROC analysis and the latter is the minimum of the ratios of the PAF group. Most statistical tests were performed with SPSS version 12.0 (SPSS Inc). The calculation and comparison of AUC values and the logistic regression analyses were performed with Stata SE version 8.2 (Stata Corp). A P value <0.05 was considered to be statistically significant. The statistical power of the present study was finally calculated.

Forty subjects were randomly selected from each group and analyzed blindly by 2 authors (N.T. and H.K.) to assess reproducibility. The interobserver and intraobserver variabilities were, respectively, 4.0% and 3.8% for LAV, 4.2% and 4.2% for LAV_{preA}, 12.3% and 9.9% for LAV_{min}, 4.4% and 4.1% for peak A velocity, 3.6% and 3.5% for septal Aa, and 4.0% and 3.7% for lateral Aa.

Table 1. Clinical Characteristics of the Study Population

Variable	Control Group (n=140)	PAF Group (n=140)
Age, y	66±7	66±8
Women, %	47	47
Height, cm	158±9	160±10
Weight, kg	58±10	60±10
Body surface area, m^2	1.59±0.17	1.60±0.26
Body mass index, kg/m^2	23.2±3.3	23.6±3.0
Systolic blood pressure, mm Hg	140±18	137±16
Diastolic blood pressure, mm Hg	82±10	80±11
Diabetes mellitus, %	17	21
Hyperlipidemia, %	46	47
Smoking, %	31	23
Concomitant cardiovascular therapies		
ACE inhibitors, %	7	5
ARBs, %	30	32
β-Blockers, %	18	27
Calcium channel blockers, %	32	37

Values are expressed as mean±SD unless otherwise specified. ACE indicates angiotensin-converting enzyme; ARB, angiotensin II receptor blockers. No significant differences were found between groups.

Results

Clinical and Echocardiographic Characteristics

A total of 280 subjects with hypertension (mean age: 66±7 years; range: 40 to 80 years; 148 men; LVEF: 65±8%) were enrolled in the present study. There were no significant differences in clinical parameters or the use of antihypertensive drugs between the 140 control and 140 PAF subjects (Table 1). The median time interval between the first PAF episode and this examination was 2.0 years (25th to 75th percentile: 0.2 to 7.0 years), and 62% of the patients with PAF were symptomatic. Echocardiographic characteristics are depicted in Table 2. No significant group differences were found for the following parameters: LV end-diastolic diameter, LV end-systolic diameter, and LVEF. LV mass index, LA dimension, and indices related to the LAV were significantly increased in the PAF group compared with those in the control group, whereas active LAEF was decreased in the PAF group. The prevalences of LV hypertrophy were 45% in the PAF group (37 women and 25 men) and 28% in the control group (18 women and 21 men). The E-wave decel-

Table 2. Echocardiographic Characteristics of the Study Population

Variable	Control Group (n=140)	PAF Group (n=140)	*P*
LV end-diastolic dimension, mm	47±5	46±5	0.114
LV end-systolic dimension, mm	28±5	28±5	0.445
LVEF, %	65±8	65±8	0.839
LV mass index, g/m^2	100±20	112±27	<0.001
LA dimension, mm	38±5	43±5	<0.001
LAVi, ml/m^2	30±7	42±12	<0.001
$LAVi_{preA}$, ml/m^2	21±6	32±10	<0.001
$LAVi_{min}$, ml/m^2	14±4	25±10	<0.001
Active LAEF, %	34±8	23±10	<0.001
Peak E velocity, cm/s	64±15	70±19	0.007
Peak A velocity, cm/s	80±18	67±21	<0.001
E/A	0.85±0.24	1.18±0.67	<0.001
E-wave deceleration time, ms	233±44	224±49	0.069
Ea, cm/s	7.5±1.9	6.8±1.9	<0.001
Aa, cm/s	11.1±2.3	7.7±2.6	<0.001
E/Ea	9.0±2.5	11.1±4.3	<0.001
LAVi/A, mL · m^{-2}/cm · s^{-1}	0.39±0.11	0.72±0.41	<0.001
LAVi/Aa, mL · m^{-2}/cm · s^{-1}	2.9±1.0	6.8±5.1	<0.001
$LAVi_{preA}$/active LAEF	0.64±0.28	1.96±2.23	<0.001
$LAVi_{preA}$/A, mL · m^{-2}/cm · s^{-1}	0.27±0.08	0.55±0.36	<0.001
$LAVi_{preA}$/Aa, mL · m^{-2}/cm · s^{-1}	1.9±0.4	5.3±4.3	<0.001

Values are expressed as mean±SD.

eration time was comparable, but peak E velocity was significantly greater, and peak A velocity was less in the PAF group than in the control group. The Ea and Aa were lower in the PAF group than in the control group. E/Ea was greater in the PAF group than in the control group.

The following correlations were found: (1) LAVi and $LAVi_{preA}$ were correlated with E/Ea (LAVi: r=0.418, P<0.001; $LAVi_{preA}$: r=0.416, P<0.001); (2) LAVi and $LAVi_{preA}$ were correlated with LV mass index (LAVi: r=0.440, P<0.001; $LAVi_{preA}$: r=0.459, P<0.001); (3) LAVi was strongly correlated with the $LAVi_{preA}$ (r=0.916; P<0.001); (4) Aa was correlated well with active LAEF (r=0.755; P<0.001) and this correlation was still observed in patients with severely enlarged LA (r=0.775; P<0.001), which is defined as LAVi >40 mL/m^2 in the recommendations[12]; and (5) LAVi/Aa and $LAVi_{preA}$/Aa were correlated with the time interval between the first PAF episode and this examination (LAVi/Aa: r=0.267, P=0.012; $LAVi_{preA}$/Aa: r=0.275, P=0.009).

Echocardiographic Detection of PAF Among Hypertensive Patients

Various parameters, listed in Table 3, were examined using ROC analysis. $LAVi_{preA}$/Aa was best for detecting patients with a history of PAF considering the AUC. The AUC of $LAVi_{preA}$/Aa was statistically greater than those of the $LAVi_{preA}$/A, $LAVi_{preA}$/active LAEF, LAVi/Aa, and LAVi values (P=0.024, P=0.003, P<0.001, and P<0.001, respectively). Although the statistical power calculated was 76% because of the limited number of subjects, statistically significant differences were found between the $LAVi_{preA}$/Aa ratio and other parameters. The ROC curves for the $LAVi_{preA}$/Aa, LAVi/Aa, LAVi, and LA dimension are shown in Figure 1.

Table 3. AUCs for Echocardiographic Variables

Variable	AUC	SE	95% CI
LA dimension	0.733	0.030	0.675 to 0.791
LAVi	0.820	0.024	0.772 to 0.868
$LAVi_{preA}$	0.861	0.022	0.819 to 0.904
LAVi/A	0.852	0.023	0.807 to 0.898
LAVi/Aa	0.884	0.019	0.847 to 0.922
$LAVi_{preA}$/active LAEF	0.893	0.019	0.856 to 0.930
$LAVi_{preA}$/A	0.888	0.020	0.849 to 0.927
$LAVi_{preA}$/Aa	0.927	0.015	0.897 to 0.956

The sensitivity, specificity, diagnostic accuracy, and positive predictive value for the detection of PAF were determined with an optimal cutoff value according to the ROC analysis, as shown in Table 4. Diagnostic accuracy and positive predictive value of the $LAVi_{preA}$/Aa ratio at the cutoff value of >2.7 mL · m^{-2}/cm · s^{-1} were clearly superior to those of the LAVi of >32.0 mL/m^2.

From the results of univariate analysis for age, sex, body weight, LV hypertrophy, E/Ea, and diabetes mellitus, LV hypertrophy (odds ratio: 2.059 [95% CI: 1.251 to 3.386]; P<0.001) and E/Ea (odds ratio: 1.205 [95% CI: 1.108 to 1.309]; P<0.001) were considered as significant covariates. However, multivariate analysis demonstrated that the $LAVi_{preA}$/Aa ratio was the single significant factor of a history of PAF (odds ratio: 11.786 [95% CI: 6.178 to 22.483]; P<0.001).

LA Size Against Atrial Pump Function

The relationship between LA size and atrial pump function in our population is illustrated in Figure 2. LA size and Aa showed an inverse correlation (LAVi: r=−0.503, P<0.001; $LAVi_{preA}$:

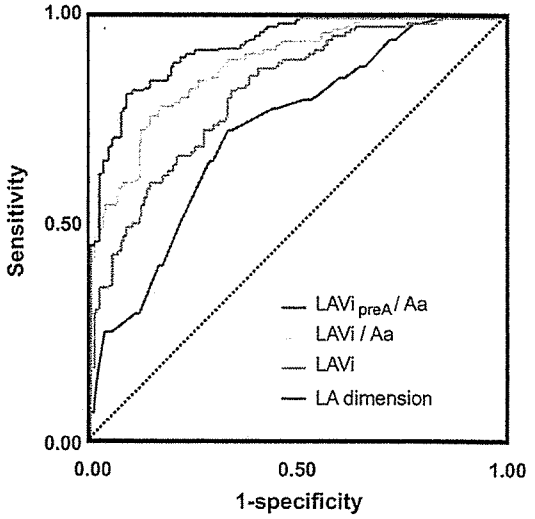

Figure 1. ROC curves for detecting PAF. Comparison of ROC curves for the $LAVi_{preA}$/Aa (red line), LAVi/Aa (yellow line), LAVi (green line), and LA dimension (blue line) values. The AUC values are listed in Table 3.

Table 4. Evaluation of Echocardiographic Parameters According to Sensitivity, Specificity, and Diagnostic Accuracy for Detection of PAF in Hypertensive Patients

Variable	Sensitivity, %	Specificity, %	Diagnostic Accuracy, %	PPV, %
LA dimension >41 mm	72	67	71	69
LAVi >32.0 mL/m^2	82	66	74	71
LAVi/A >0.50 mL · m^{-2}/cm · s^{-1}	75	87	81	85
LAVi/Aa >3.6 mL · m^{-2}/cm · s^{-1}	78	84	82	81
LAVi$_{preA}$/A >0.36 mL · m^{-2}/cm · s^{-1}	80	85	83	84
LAVi$_{preA}$/Aa >2.7 mL · m^{-2}/cm · s^{-1}	82	91	86	90

Values are expressed as percentage. PPV indicates positive predictive value.

$r=-0.458$, $P<0.001$). The PAF group is located disproportionately in the upper left part as compared with the control group. The black dotted lines discriminate best between the PAF and control groups, with slopes of 3.6 (the LAVi/Aa ratio=3.6 mL · m^{-2}/cm · s^{-1}) and 2.7 (the LAVi$_{preA}$/Aa ratio=2.7 mL · m^{-2}/cm · s^{-1}), respectively. The red dotted lines show the lower bound of the PAF group, with slopes of 2.3 and 1.9, respectively.

Discussion

The LA of hypertensive patients with PAF was characterized by further enlargement and impaired pump function as compared with that of hypertensive patients in whom PAF had never been documented. Thus, the ratio of LAVi$_{preA}$ to Aa was most powerful, and the ratio of LAVi to Aa was the next most powerful for differentiating hypertensive patients with a history of PAF.

LA Remodeling in Hypertension

LA size has been reported to serve as a surrogate measure of chronic LV diastolic dysfunction.[27] Hypertension induces an increase in LV wall stress, leading to increased wall thickness, myocyte hypertrophy, and myocardial fibrosis.[28,29] Impaired myocardial relaxation and increased LV diastolic stiffness can cause elevated LV diastolic filling pressure,[30] consistent with the increase in E/Ea. Moreover, long-standing hypertension results in interstitial fibrosis and arrhythmic substrate even in the LA.[31] In a large cohort study, increased LV mass and LA diameter were independently associated with the occurrence of AF in patients with hypertension.[32] Similarly, we found that LV mass index, E/Ea, and LAVi values were significantly increased in our hypertensive patients with PAF compared with those in hypertensive patients without PAF. In addition, LAVi showed a significant correlation with both LV mass index and E/Ea. These findings do not contradict the concept that the occurrence of PAF in hypertension is associated with LA remodeling as a consequence of LA overload because of elevated LV filling pressure.

The LAVi$_{preA}$/Aa and LAVi/Aa ratios showed a very modest but yet significant correlation with the time interval between the first PAF episode and this examination. LA remodeling is thought to progress according to not only the LA overload but also the duration of AF.[33] Thus, the indices that we proposed may be markers representing the degrees of progressive LA remodeling.

Frank-Starling Mechanism of LA Function

According to the Frank-Starling mechanism, contractile force of the ventricular myocardium is proportional to its initial length; this mechanism also applies to the LA myocardium.[22,23] LA pump function is enhanced in response to elevated LV filling pressure as long as the Frank-Starling mechanism holds. Within the range of compensation, therefore, the points in Figure 2 should range toward the upper right direction; in contrast, the points belonging to the PAF group are located in the upper left area. This disproportional distribution is also thought to demonstrate a shift of or a deviation from the Frank-Starling curves because of further progression of atrial remodeling in the PAF group. In addition, Figure 2 shows that patients with an LAVi/Aa ratio ≥2.3 mL · m^{-2}/cm · s^{-1}

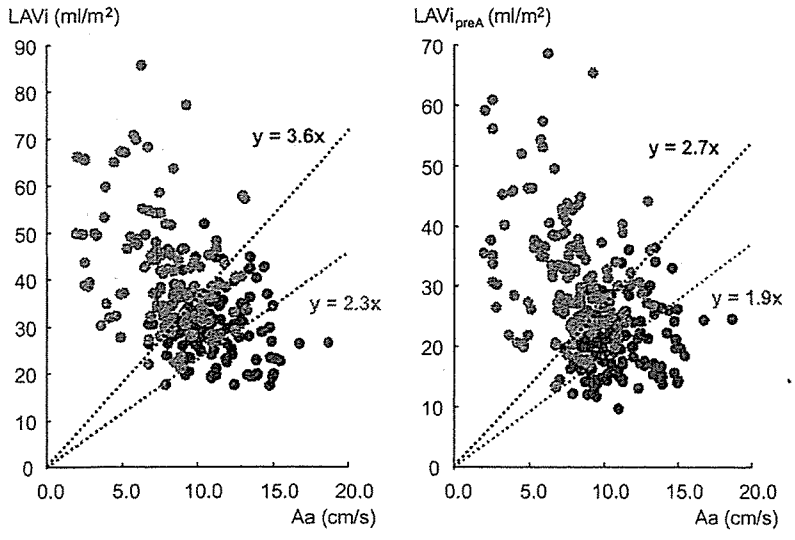

Figure 2. Relationship between LA size and pump function in patients with and without PAF. Scatter diagrams showing the distribution of plots of the LAVi against Aa values (left) and LAVi$_{preA}$ against Aa values (right). Red points indicate hypertensive patients with PAF, and blue points indicate hypertensive patients in whom PAF has never been documented. Patients with LAVi/Aa ratios >2.3 mL · m^{-2}/cm · s^{-1} or LAVi$_{preA}$/Aa ratios >1.9 mL · m^{-2}/cm · s^{-1} may have PAF.

or $LAVi_{preA}/Aa$ ratio ≥ 1.9 mL · m^{-2}/cm · s^{-1} may potentially have a history of PAF.

Preload is originally reflected as the initial stretching of cardiac myocytes before contraction. Thus, when adding atrial function to LA size for assessing atrial remodeling, $LAVi_{preA}$ is thought to be physiologically more preferable to LA size than is LAVi. Therefore, it was a reasonable result that the $LAVi_{preA}/Aa$ ratio showed better diagnostic ability than the LAVi/Aa ratio.

Measurement of Atrial Pump Function

Several past studies have demonstrated that Aa velocity correlates well with atrial contractile function and can be used as a rapid and accurate marker of atrial function.[16–20] Similar to results of these studies, Aa showed a significant correlation with active LAEF in our study, and the correlation of Aa with active LAEF also remained in patients with excessive LA enlargement.

The changes in flow velocity profile with different positions of the pulsed-wave Doppler sample volume may affect the diagnostic accuracy of the $LAVi_{preA}/A$ ratio. Even slight changes in sample volume position can easily cause a decrease in peak A velocity.[34] In contrast, TDI-derived mitral annulus velocities are relatively independent of these problems.[35] Our results also show that Aa is a marker reflecting atrial pump function.

Lateral Aa is a favorable discrimination parameter because its amplitude is greater than that of septal Aa. However, the left lung often attenuates tissue Doppler signals from the lateral annulus margin. The mitral septal annulus motion is always parallel to the Doppler beam, and its measurements are certain. Septal Aa could, however, be influenced by right atrial pump function. In the present study, therefore, we used the average of the septal and lateral Aas.

Study Limitations

First, this was a cross-sectional study. To clarify whether the present index can predict future AF development or complications, further research must provide longitudinal assessments. Second, it is difficult to distinguish whether the impaired atrial pump function was attributed to LA remodeling or showed a recovery process after spontaneous conversion of AF. This issue does not concern the diagnosis of PAF, but it may slightly affect the cutoff value for detecting PAF. Third, atrial reservoir function was not examined in the present study. Some reports indicate that evaluating LA reservoir function using strain or strain rate is useful for the prediction of AF relapse after treatments.[36,37] Nevertheless, we chose to use pulsed TDI parameters for the following reasons: (1) measurement of strain or strain rate with high reproducibility or using the speckle tracking method requires a high-end ultrasound machine and special dedicated software, but pulsed TDI is available on almost all machines; (2) peak late-diastolic atrial strain rate during atrial contraction has been reported to be correlated well with Aa[38,39]; (3) speckle tracking software, which is used when measuring atrial strain or strain rate, is designed primarily for the LV and not the LA; and (4) atrial strain or strain rate reflects only regional function of the LA, and the position of regions of interest for assessing LA global pump function is still controversial, whereas Aa is a marker of atrial global function and has been validated by cardiac catheters.[17] We preferred this general technology because our goal was to make our method accessible. Fourth, we did not measure pulmonary venous flow for assessing LV diastolic function because obtaining an adequate signal for analysis has been reported to be difficult in all subjects.[40,41] Furthermore, a recent consensus statement recommends tissue Doppler velocities as a first-line echocardiographic diagnostic approach to LV diastolic dysfunction.[42] Fifth, obtaining an accurate time interval between the first PAF episode and the current examination was practically difficult because PAF is, by nature, an elusive disease, and the majority of PAF episodes are known to be asymptomatic.[43] Similarly, the first PAF episode in asymptomatic patients was noticed incidentally as an irregularly irregular rhythm during auscultation or palpitating arterial pulse and was confirmed using ECG at a monthly or biweekly consultation day; thus, the interval may be not accurate. Little information on the number and duration of PAF episodes was available in the present study. Finally, we cannot clearly state that self-terminating spontaneous AF was never present in hypertensive patients with PAF within 1 week of examination or in patients with hypertension alone; nonetheless, we addressed this concern by performing repeated 12-lead ECGs, 24-hour Holter monitoring, and strict medical interviews for the subjects of the study.

Perspectives

Our observations that the LA of hypertensive patients with PAF is characterized by dilatation and impaired pump function and that the ratio of LA size to pump function is useful for identifying patients with PAF have important public health and clinical implications. If the presence of PAF is suspected at the time of echocardiography, further examinations and careful monitoring (including repeated 24-hour or event-ECG Holter recording) may be considered. This is especially true for patients with multiple risk factors for stroke (eg, congestive heart failure, hypertension, age >75 years, diabetes mellitus, and previous stroke or transient ischemic attack). In other words, the present indices may be useful for recommending preventive therapy in high-risk patients, because clinicians can prevent the greater part of ischemic stroke from AF with anticoagulation therapy.[44] A recent report from the Framingham Heart Study proposed a risk score for the future development of AF, but echocardiographic parameters provided only slight improvement in the risk assessment score.[45] The measurements derived from the conventional M-mode method may have attenuated the benefits of echocardiography in the aforementioned study. We expect that the new marker presented here (LA size divided by atrial pump function) will improve risk classification in future prospective studies.

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Disclosures

None.

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Common Variants in the ATP2B1 Gene Are Associated With Susceptibility to Hypertension

The Japanese Millennium Genome Project

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Abstract—Hypertension is one of the most common complex genetic disorders. We have described previously 38 single nucleotide polymorphisms (SNPs) with suggestive association with hypertension in Japanese individuals. In this study we extend our previous findings by analyzing a large sample of Japanese individuals (n=14 105) for the most associated SNPs. We also conducted replication analyses in Japanese of susceptibility loci for hypertension identified recently from genome-wide association studies of European ancestries. Association analysis revealed significant association of the *ATP2B1* rs2070759 polymorphism with hypertension ($P=5.3\times10^{-5}$; allelic odds ratio: 1.17 [95% CI: 1.09 to 1.26]). Additional SNPs in *ATP2B1* were subsequently genotyped, and the most significant association was with rs11105378 (odds ratio: 1.31 [95% CI: 1.21 to 1.42]; $P=4.1\times10^{-11}$). Association of rs11105378 with hypertension was cross-validated by replication analysis with the Global Blood Pressure Genetics consortium data set (odds ratio: 1.13 [95% CI: 1.05 to 1.21]; $P=5.9\times10^{-4}$). Mean adjusted systolic blood pressure was highly significantly associated with the same SNP in a meta-analysis with individuals of European descent ($P=1.4\times10^{-18}$). *ATP2B1* mRNA expression levels in umbilical artery smooth muscle cells were found to be significantly different among rs11105378 genotypes. Seven SNPs discovered in published genome-wide association studies were also genotyped in the Japanese population. In the combined analysis with replicated 3 genes, *FGF5* rs1458038, *CYP17A1*, rs1004467, and *CSK* rs1378942, odds ratio of the highest risk group was 2.27 (95% CI: 1.65 to 3.12; $P=4.6\times10^{-7}$) compared with the lower risk group. In summary, this study confirmed common genetic variation in *ATP2B1*, as well as *FGF5*, *CYP17A1*, and *CSK*, to be associated with blood pressure levels and risk of hypertension. (***Hypertension*. 2010;56:973-980.**)

Key Words: hypertension ■ genetic variation ■ ATP2B1 ■ Millennium Genome Project ■ Global BPgen

Because of its large impact on a number of cardiovascular diseases, hypertension is a major contributor to global health burden. Because hypertension is one of the most prevalent complex genetic disorders, with a heritability of ≤60% based on the estimation by 24-hour blood pressure (BP) readings,[1] numerous studies, including recent genome-wide association studies (GWAS),[2–6] have attempted to identify genetic variation associated with human BP levels.

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Except for rare mendelian forms of hypertension,[7] the estimated effects of each genetic factor on BP levels have been found to be small in the general population (typically <1.0 mm Hg on systolic BP [SBP] and <0.5 mm Hg on diastolic BP [DBP] per risk allele). However, multiple risk alleles are known to have a cumulative impact on several complex traits, including BP and hypertension risk.[3] In addition, it is anticipated that identification of novel susceptibility genes would lead to further understanding of disease pathogenesis.

As a part of a series of nationally based cooperative projects, the Millennium Genome Project (Millennium GPJ), we conducted multiple candidate gene analyses to identify susceptible genes and polymorphisms for hypertension. In a previously reported study,[6] we focused on 307 genes, which were genes encoding components of signal transduction pathways potentially related to BP regulation, including receptors, soluble carrier proteins, binding proteins, channels, enzymes, and G proteins. That study identified 38 single nucleotide polymorphisms (SNPs) as suggestively associated with hypertension by analysis of 758 hypertensive patients and 726 normotensive controls.[6] To extend our previous study, we have now genotyped all 38 of the SNPs in a replication panel composed of 1929 hypertensives and 1993 normotensives and have taken forward validated SNPs with further genotyping in a large Japanese genetic epidemiological cohort sample (n=14 105). An in silico validation analysis of our most promising loci was performed using the Global Blood Pressure Genetics (Global BPgen) consortium data set, a large-scale GWAS of samples of European descent.[2] Furthermore, we also conducted a replication analysis of recent European GWAS-derived susceptible loci for hypertension from Global BPgen[2] and CHARGE (Cohorts for Heart and Aging Research in Genome Epidemiology) GWAS[3] in a Japanese large-scale general population sample (Figure S1, available in the online Data Supplement at http://hyper.ahajournals.org).

Methods

Case and Control Subjects (Screening Panel)

Details of the screening panel subjects have been described previously.[6] Briefly, hypertensive patients and normotensive controls were recruited in the Asahikawa, Tokyo, Osaka, and Hiroshima regions of Japan according to the following criteria. Hypertensive subjects (n=758) had a previous diagnosis of hypertension at between 30 and 59 years of age and were either being treated with antihypertensive medication or had a SBP >160 mm Hg and/or DBP >100 mm Hg. They had a family history of hypertension in their parents and/or siblings and were not obese (body mass index [BMI] <25 kg/m^2). Normotensive controls (n=726) aged >45 years were recruited from the same regions. These individuals have never been treated with antihypertensive medications, and their SBP was <120 mm Hg and DBP <80 mm Hg. They had no family history of hypertension. All of the subjects were unrelated and were native Japanese.

Cohort-Based Population Samples

Seven independent study cohorts for cardiovascular diseases and related risk factors were combined to compose a large-scale Japanese genetic epidemiological population sample of 14 105. The Ohasama, Shigaraki, Takashima, Suita, and Nomura studies are general population-based genetic epidemiological studies. The study subjects were recruited via a medical checkup process for community residents. The 2 other cohorts, Yokohama and Matsuyama, are derived from employees of large manufacturing industries. The clinical parameters used in this study were obtained from personal health records during annual medical checkups. Further details of the study cohorts are described in the online Data Supplement.

Nested Case and Control Subjects Derived From the Cohort-Based Sample (Replication Panel)

Hypertensive cases and normotensive controls were chosen from the cohort-based population samples described above (n=11 569; the Suita study was excluded because of ethical issues). The selection criteria of the hypertensive and normotensive subjects were as follows: hypertensive subjects (n=1929) aged ≤64 years and either treatment with antihypertensive medication and/or SBP >160 mm Hg and/or DBP >90 mm Hg; normotensive subjects (n=1993) aged ≥40 years and having SBP <120 mm Hg and DBP <80 mm Hg; and no current use of antihypertensive medication and free from any history of cardiovascular disease.

Global BPgen (In Silico) Analyses

To investigate cross-validation of the most promising SNPs, we obtained results for 4 SNPs in the *ATP2B1* gene from the Global BPgen consortium, a study that is composed of 17 GWAS studies with 34 433 individuals of European descent. A detailed description of the study design and phenotype measurement for all of the cohorts has been reported previously.[2]

Validation of Published BP Polymorphisms in the Japanese Millennium Cohort

Thirteen loci have been identified recently and robustly validated for association with BP and hypertension in recent large-scale GWAS of European samples, by the Global BPgen consortium[2] and the CHARGE consortium.[3] From the associated SNPs reported at these 13 loci, we selected SNPs expected to have minor allele frequencies in Japanese samples >0.10, based on the HapMap database (JPT only, Public Release No. 27)[8]: *FGF5* rs1458038, *CYP17A1* rs1004467, *CSK* rs1378942, *PLCD3* rs12946454, *PLEKHA7* rs381815, *ULK4* rs9815354, and *CSK-ULK3* rs6495122. These 7 SNPs were genotyped in the Japanese population-based cohort sample to test whether the same associations exist in samples of Japanese ancestry.

Genotyping

Genomic DNA was extracted from peripheral blood. All of the SNPs were analyzed by TaqMan probe assays (Applied Biosystems Co, Ltd) using commercially available primers and probes purchased from the Assay-on-Demand system. The fluorescence level of PCR products was measured using an ABI PRISM 7900HT sequence detector.

Ethical Considerations

All of the study procedures were approved by the ethics committee of each university or research institute. Written informed consent was obtained from all of the participating subjects.

Ex Vivo Expression Analysis of ATP2B1 mRNA

Umbilical artery smooth muscle cells were isolated from umbilical cords obtained at delivery (n=34). Expression levels of ATP2B1 mRNA were analyzed by RT-PCR using a relative quantification method. Further details of the ex vivo expression analysis are described in the online Data Supplement.

Statistical Analysis

At each SNP, frequency differences in each genotype among hypertensive and normotensive subjects were assessed using a χ^2 test. Linkage disequilibrium (LD) coefficients were calculated using the Haploview software (Broad Institute).[9] Adjusted odds ratios for hypertension, as well as coefficients and SEs for SBP and DBP, were calculated using logistic and linear multiple regression analysis,

Table 1. Association of ATP2B1 SNPs With Hypertension in the Screening and Replication Panels

			Screening Panel						Replication Panel						
SNP	Genotype		Genotype Frequency			HWE	Call Rate	Odds (*P*)	Genotype Frequency			HWE	Call Rate	Odds (*P*)	Overall Odds (*P*)
rs1401982	AA/AG/GG	HT	318	328	92	0.603	96.3	1.28 (0.001)	825	833	247	0.108	98.7	1.25 (3.0×10^{-6})	1.26 (1.5×10^{-8})
		NT	249	324	118	0.474			699	961	305	0.397			
rs2681472	AA/AG/GG	HT	335	321	90	0.334	97.8	1.26 (0.003)	846	832	242	0.095	99.5	1.26 (1.0×10^{-6})	1.26 (8.7×10^{-9})
		NT	267	328	111	0.539			715	966	303	0.431			
rs2070759	GG/GT/TT	HT	216	379	151	0.515	97.6	1.16 (0.045)	582	896	399	0.118	97.2	1.18 (4.4×10^{-4})	1.17 (5.3×10^{-5})
		NT	186	341	175	0.454			507	956	474	0.579			
rs11105364	TT/TG/GG	HT	335	322	88	0.432	97.2	1.29 (0.001)	846	834	236	0.171	99.3	1.25 (2.4×10^{-6})	1.26 (4.1×10^{-9})
		NT	261	323	113	0.438			729	947	303	0.874			
rs11105378	CC/CT/TT	HT	359	301	76	0.276	97.3	1.37 (6.3×10^{-5})	868	821	217	0.280	98.8	1.28 (1.4×10^{-7})	1.31 (4.1×10^{-11})
		NT	280	320	108	0.295			746	922	300	0.586			

The screening panel is composed of 758 middle age–onset severe hypertensive patients and 726 middle-aged to elderly evidently normotensive controls (Table S4). The replication panel consists of 1929 hypertensive cases, and 1993 normotensive controls selected from 11 569 cohort sample were enrolled (Table S2). ORs and *P* values for allelic model are shown.

adjusting for sex, age, age^2, BMI, and cohort variables, using additive (1 degree of freedom) and genotypic (2 degrees of freedom) genetic models. Adjustment for treatment with antihypertensive medication was achieved by adding fixed constants to measured values (+15 mm Hg for SBP and +10 mm Hg for DBP).[10] The Global BPgen data and statistical methods have been described elsewhere.[2] Meta-analysis was performed assuming fixed effects and using inverse variance weights. An unweighted genetic risk score based on 4 SNPs (*ATP2B1* rs1105378, *FGF5* rs1458038, *CYP17A1* rs1004467, and *CSK* rs1378942) was calculated by adding the number of risk alleles showing higher BP values. Risk allele of each SNP was defined as follows: *ATP2B1*, C allele; *FGF5*, T allele; *CYP17A1*, A allele; and *CSK*, C allele. The *CSK-ULK3* SNP rs6495122 showing positive association with BP trait and hypertension was not included in the calculation of genetic risk score, because the strong LD with the *CSK* SNP rs1378942 ($D'=0.884$; $r^2=0.731$) is most parsimoniously explained by both SNPs tagging a single risk variant. Differences in mRNA expression levels among the *ATP2B1* rs1105378 genotype were assessed by ANOVA. The statistical analyses were performed using a commercially available statistical software package (JMP version 8, SAS Institute).

Results

Replication Genotyping

The clinical characteristics of the replication panel chosen from the cohort-based population samples (Table S1, available in the online Data Supplement) are shown in Table S2. Stringent case and control definitions, corresponding with the extreme upper ≈17% and lower ≈17% of the general population, were used to maximize power for fixed genotyping costs.[11] Thirty-six SNPs were successfully genotyped, and results for all of the SNPs are shown in Table S3. Significant association was observed for the *ATP2B1* rs2070759 polymorphism located in intron 8 ($P=4.4\times10^{-4}$; allele odds ratio [OR]: 1.18 [95% CI: 1.07 to 1.29]). Several other SNPs also showed marginally significant association; however, the *P* values did not reach statistical significance after application of Bonferroni correction for multiple comparisons (threshold: 0.05/36=0.0014; Table S3; we note that no other SNPs are significant if the less conservative Benjamini-Hochberg procedure is used to control the false discovery rate at 0.05). Although, the replication results in the less-strict nested case-control sample chosen from the same population sample have been reported in our previous article,[6] the association was recalculated to narrow down the SNPs to be applied to the following dense SNP analysis.

Dense SNP Analysis of the ATP2B1 Gene

To more precisely identify the SNP or SNPs increasing susceptibility for hypertension, we performed "de novo" genotyping of a dense SNP panel around marker rs2070759 in individuals from the original screening panel (Table S4).[6] Forty-one tag SNPs located in a 167-kb region around rs2070759 were selected using the HapMap database (Table S5).[8] Among the 27 SNPs polymorphic in our Japanese sample, the most significant association was observed with rs11105378; this yielded an allelic *P* value of 6.3×10^{-5} (OR: 1.37 [95% CI: 1.17 to 1.60]; Table 1 and Figure S2).

The most associated SNP and the 4 others from the dense SNP analyses were subsequently genotyped in the replication panel. Significant association of rs11105378 was confirmed in the replication panel with an allelic *P* value of 1.4×10^{-7} (OR: 1.28 [95% CI: 1.17 to 1.41]; Table 1). Meta-analysis of both study panels indicated significant association ($P=4.1\times10^{-11}$; OR: 1.31 [95% CI: 1.21 to 1.42]) and confirmed that the strongest association is seen for rs11105378. The D′ and r^2 measures of LD between rs2070759 and rs11105378 were 0.92 and 0.48, respectively. Other SNPs, rs1401982 ($D'=0.99$; $r^2=0.64$), rs2681472 ($D'=0.99$; $r^2=0.61$), rs11105364 ($D'=0.97$; $r^2=0.59$), located within the same LD block, were also significantly associated with hypertension (Table 1). The strong LD between associated SNPs suggests a single true association signal in this region.

We examined for possible association of SNPs in the *ATP2B4* gene, a well-investigated isoform of the *ATP2B1* gene, with hypertension in the screening panel. We observed no significant correlation with the 17 SNPs analyzed, which were selected using the HapMap database (Table S6).

Population-Based Meta-Analyses of ATP2B1 SNPs

The complete Japanese population-based sample was subsequently genotyped for the 4 most significant SNPs in

Table 2. Meta-Analysis of ATP2B1 SNPs With BP Traits

		Millennium GPJ			Global BPgen			CHARGE*			Pooled	
SNP	Coded Allele	n (Frequency)	Coefficient (SE), mm Hg	*P*	n (Frequency)	Coefficient (SE), mm Hg	*P*	n (Frequency)	Coefficient (SE), mm Hg	*P*	Coefficient (95% CI), mm Hg	*P*
SBP												
rs1401982	G	13 944	−1.22	1.8×10^{-7}	33 885	−0.30	0.022				−0.52	3.9×10^{-6}
		(0.376)	(0.23)		(0.385)	(0.13)					(−0.74 to −0.30)	
rs2681472	G	14 032	−1.33	1.2×10^{-8}	33 803	−0.62	5.2×10^{-4}	0.17	−1.29	3.5×10^{-11}	−1.03	9.9×10^{-20}
		(0.373)	(0.23)		(0.158)	(0.18)			(0.19)		(−1.26 to −0.81)	
rs11105364	G	14 013	−1.34	8.9×10^{-9}	33 877	−0.60	7.4×10^{-4}	0.17	−1.30	4.8×10^{-11}	−1.03	1.2×10^{-19}
		(0.364)	(0.23)		(0.179)	(0.18)			(0.19)		(−1.25 to −0.81)	
rs11105378	T	13 948	−1.33	1.5×10^{-8}	33 171	−0.59	0.001	0.16	−1.31	9.1×10^{-11}	−1.02	1.4×10^{-18}
		(0.360)	(0.23)		(0.158)	(0.18)			(0.20)		(−1.24 to −0.79)	
DBP												
rs1401982	G	13 944	−0.72	2.0×10^{-7}	33 898	−0.18	0.041				−0.34	8.1×10^{-6}
		(0.376)	(0.14)		(0.392)	(0.09)					(−0.49 to −0.19)	
rs2681472	G	14 032	−0.65	2.7×10^{-6}	33 829	−0.35	0.003	0.17	−0.64	3.7×10^{-8}	−0.54	9.7×10^{-15}
		(0.373)	(0.14)		(0.157)	(0.12)			(0.11)		(−0.68 to −0.41)	
rs11105364	G	14 013	−0.70	4.5×10^{-7}	33 898	−0.34	0.004	0.17	−0.63	1.2×10^{-7}	−0.54	7.5×10^{-14}
		(0.364)	(0.14)		(0.158)	(0.12)			(0.12)		(−0.68 to −0.40)	
rs11105378	T	13 948	−0.70	5.4×10^{-7}	33 183	−0.33	0.005	0.16	−0.62	3.1×10^{-7}	−0.54	1.6×10^{-13}
		(0.360)	(0.14)		(0.158)	(0.12)			(0.12)		(−0.68 to −0.39)	

Coefficients and SE for SBP and DBP were calculated under the additive model using multiple regression analysis adjusted for age, age^2, sex, and BMI. In both Millennium GPJ and Global BPgen, adjustment for treatment with antihypertensive medication was achieved by adding fixed constants to measured values (+15 mm Hg for SBP and +10 mm Hg for DBP).[2] In the Japanese Millennium GPJ and also for some cohorts within Global BPgen, cohort variables were also adjusted to avoid residual population stratification.

*Results of the CHARGE Study were obtained from the published article.[3]

ATP2B1. To further validate and get more precise effect size estimates in Japanese, for this analysis, hypertensive cases were defined as individuals with treatment with antihypertensive medication, SBP ≥140 mm Hg, or DBP ≥90 mm Hg. The ORs for the 4 SNPs were all extremely similar (ranging from 1.19 to 1.21 under the additive model adjusted for age, age^2, sex, BMI, and cohort variables; see Table S7). These associations were replicated in the Global BPgen subjects of European descent; the pooled analysis demonstrated increased significance (rs1105378: OR: 1.17 [95% CI: 1.11 to 1.23]; $P=7.0\times10^{-10}$), as expected for a larger total sample size (n=28 866; Table S7).

We next evaluated the effect of the most associated SNP, rs11105378, on BP levels in the Millennium GPJ cohort (Table 2). We adjusted for several covariates that are associated with BP phenotypes: age ($r=0.362$; $P<0.001$ for SBP), BMI ($r=0.275$; $P<0.001$), and sex (male: 131.7±18.2; female: 128.6±20.8 mm Hg; $P<0.001$). In multiple regression analysis for BP levels, including also cohort indicator variables as covariates, the results for a 2-degree-of-freedom test with the TT genotype as a reference identified both the TC genotype (coefficient=+1.66 mm Hg; $P=2.2\times10^{-4}$) and CC genotype (+2.47 mm Hg; $P=4.9\times10^{-8}$) as independent determinants for SBP after adjustment. The TC (+0.91 mm Hg; $P=8.0\times10^{-4}$) and CC genotypes (+1.32 mm Hg; $P=1.8\times10^{-6}$) were also independently associated with DBP levels. We depict the covariate adjusted mean BP levels by rs11105378 genotype in Figure S3. Results of each cohort separately are summarized in Table S8. We next performed a meta-analysis of data from the Millennium GPJ and 2 large epidemiological studies (Global BPgen and CHARGE; Table 2). Results show the per-allele differences in SBP and DBP to be ≈1.0 and 0.5 mm Hg, respectively.

Genotype-Specific Differences in Ex Vivo Expression of ATP2B1 mRNA

Differences in *ATP2B1* mRNA expression in umbilical artery smooth muscle cells among rs11105738 genotype are shown in Figure 1. Assuming a recessive genetic model, cells homozygous for T allele showed significantly higher levels of

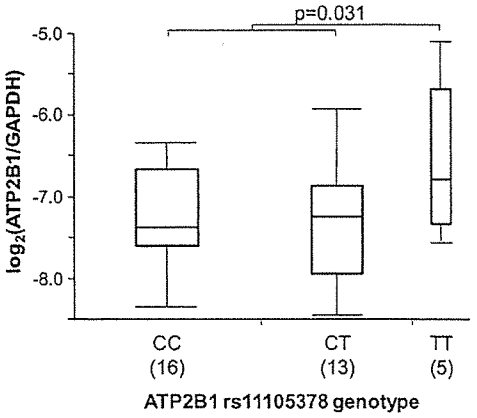

Figure 1. Ex vivo expression analysis of *ATP2B1* mRNA. Graphs depict the log^2 relative expression levels of the *ATP2B1* mRNA in umbilical artery smooth muscle cells obtained by normalizing to GAPDH. Genotype of *ATP2B1* rs11105378 of each sample was analyzed by direct sequencing using isolated genomic DNA from umbilical artery smooth muscle cells.

Table 3. Meta-Analysis of SNPs With BP Traits

SNP	Coded Allele	Millennium GPJ n (Frequency)	Millennium GPJ Coefficient (SE), mm Hg	Millennium GPJ P	Global BPgen n (Frequency)	Global BPgen Coefficient (SE), mm Hg	Global BPgen P	Pooled Coefficient (95% CI), mm Hg	Pooled P
Systolic BP									
FGF5	T	13 826	1.33	1.6×10^{-8}	30 850	0.62	1.6×10^{-6}	0.81	1.1×10^{-11}
rs1458038		(0.343)	(0.23)		(0.275)	(0.14)		(0.58 to 1.05)	
CYP17A1	A	14 007	0.89	2.3×10^{-4}	33 735	0.94	1.0×10^{-5}	0.92	6.2×10^{-9}
rs1004467		(0.680)	(0.24)		(0.901)	(0.21)		(0.61 to 1.23)	
CSK	C	13 920	0.77	0.007	34 126	0.62	2.4×10^{-6}	0.65	4.2×10^{-8}
rs1378942		(0.803)	(0.28)		(0.36)	(0.13)		(0.42 to 0.88)	
PLCD3	T	14 003	0.11	0.703	32 120	0.68	3.9×10^{-6}	0.57	2.5×10^{-5}
rs12946454		(0.831)	(0.30)		(0.28)	(0.15)		(0.30 to 0.83)	
PLEKHA7	T	14 030	0.11	0.687	33 706	0.52	2.6×10^{-4}	0.44	4.7×10^{-4}
rs381815		(0.199)	(0.28)		(0.26)	(0.14)		(0.19 to 0.68)	
CSK-ULK3	A	14 014	0.68	0.017	33 308	0.47	2.4×10^{-4}	0.51	1.7×10^{-5}
rs6495122		(0.812)	(0.28)		(0.45)	(0.13)		(0.28 to 0.74)	
ULK4	A	13 976	−0.67	0.059	32 034	0.17	0.297	0.01	0.950
rs9815354		(0.116)	(0.35)		(0.18)	(0.17)		(−0.29 to 0.31)	
DBP									
FGF5	T	13 826	0.73	1.8×10^{-7}	30 850	0.55	1.5×10^{-8}	0.61	6.1×10^{-14}
rs1458038		(0.343)	(0.14)		(0.275)	(0.10)		(0.45 to 0.77)	
CYP17A1	A	14 007	0.29	0.047	33 735	0.40	5.4×10^{-3}	0.35	4.9×10^{-4}
rs1004467		(0.680)	(0.14)		(0.901)	(0.14)		(0.15 to 0.54)	
CSK	C	13 920	0.41	0.015	34 126	0.48	5.9×10^{-8}	0.46	5.2×10^{-9}
rs1378942		(0.803)	(0.17)		(0.36)	(0.09)		(0.31 to 0.62)	
PLCD3	T	14 003	0.14	0.426	32 120	0.34	5.7×10^{-4}	0.30	1.9×10^{-4}
rs12946454		(0.831)	(0.18)		(0.28)	(0.09)		(0.14 to 0.46)	
PLEKHA7	T	14 030	0.13	0.437	33 706	0.23	0.014	0.20	0.018
rs381815		(0.199)	(0.17)		(0.26)	(0.10)		(0.04 to 0.37)	
CSK-ULK3	A	14 014	0.38	0.027	33 308	0.35	4.2×10^{-5}	0.36	7.4×10^{-6}
rs6495122		(0.812)	(0.17)		(0.45)	(0.09)		(0.20 to 0.51)	
ULK4	A	13 976	0.21	0.325	32 034	0.40	2.9×10^{-4}	0.36	2.3×10^{-4}
rs9815354		(0.116)	(0.21)		(0.18)	(0.11)		(0.17 to 0.55)	

ATP2B1 mRNA as compared with cells carrying 1 or 2 C alleles ($P=0.031$; see Figure 1). Under an additive genetic model, the overall P value was marginally significant ($P=0.091$).

Replication Analysis of European GWAS-Derived Susceptible SNPs in Japanese

We next conducted a replication analysis in the Millennium GPJ, in which we tested associated SNPs identified in recent large-scale European GWAS by the Global BPgen[2] and the CHARGE consortia.[3] From the 7 most promising SNPs of which the minor allele frequency in Japanese was >0.10 based on the HapMap database, 4 SNPs, namely, *FGF5* rs1458038, *CYP17A1* rs1004467, *CSK* rs1378942, and *CSK-ULK3* rs6495122, showed significant association in either binary trait analyses (Tables S9) or quantitative trait analysis (Table 3 and S10). The most significant association was observed with *FGF5* rs1458038; this yielded a P value of 1.6×10^{-8} (+1.33 mm Hg) with SBP and 1.8×10^{-7} (+0.73 mm Hg) with DBP in the Millennium GPJ cohort, and the effect size was greater than that of Europeans (Table 3). Meta-analysis of both study panels with data from Global BPgen indicated further significant associations.

Multiple Regression Analysis for BP Trait and Hypertension in Japanese

To clarify whether the 4 susceptibility SNPs (*ATP2B1*, *FGF5*, *CYP17A1*, and *CSK*) were independently associated with BP traits and hypertension, multiple regression analysis was performed with possible covariates (Table S11). After adjustment for age, age^2, sex, BMI, and drinking habits, this analysis confirmed that all 4 of the SNPs were independent determinants for both BP traits and hypertension.

Combined Effect of Risk Genotypes on Hypertension

A risk score for 4 susceptible genotypes was calculated to evaluate their combined effects on hypertension. ORs asso-

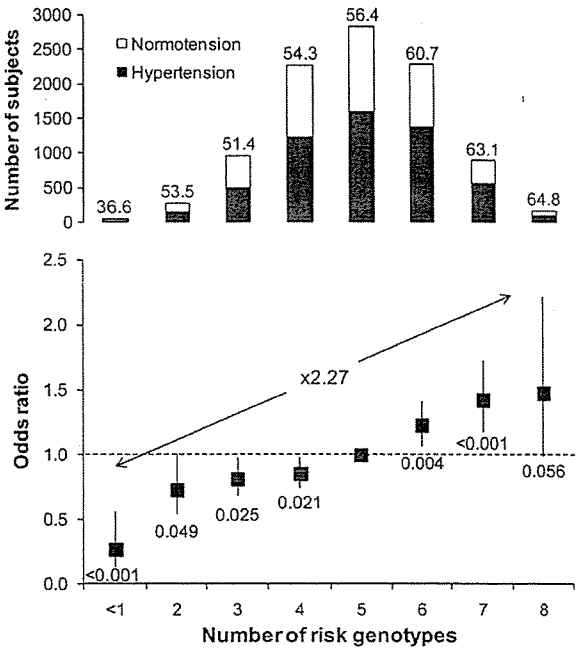

Figure 2. ORs for hypertension according to the number of risk genotypes Number of risk genotype was calculated by the following 4 SNPs: *ATP2B1* rs1105378, *FGF5* rs1458038, *CYP17A1*, rs1004467, and *CSK* rs1378942. Hypertensive subjects were defined as being treated with antihypertensive medication, SBP ≥140 mm Hg, or DBP ≥90 mm Hg; normotensive subjects were defined as all not treated with antihypertensive medication, SBP ≤120 mm Hg, and DBP ≤85 mm Hg.[2] Adjusted OR for hypertension and BP levels were calculated using logistic and linear multiple regression analysis, adjusting for sex, age, age^2, BMI, and cohort variables. Frequency of hypertension and *P* values for the hypertension odds are shown in the top of column and the bottom of square, respectively.

ciated with increasing number of risk genotypes in a covariates adjusted logistic regression model are depicted in Figure 2 (overall P value was 5.4×10^{-5}). Compared with the reference group (5 risk genotypes), individuals carrying 7 or 8 risk genotypes had higher risk (OR: 1.43 [95% CI: 1.20 to 1.72]; $P=1.0\times10^{-4}$) in contrast to the lower OR of individuals with ≤2 risk genotypes (OR: 0.63 [95% CI: 0.47 to 0.85]; $P=0.020$). The OR of the high-risk group was raised to 2.27 (95% CI: 1.65 to 3.12; $P=4.6\times10^{-7}$) compared with the lowest risk group. Adjusted per-allele OR for hypertension was 1.17 (95% CI: 1.12 to 1.21; $P=4.0\times10^{-15}$). The distribution of the Japanese population sample among the number of risk genotypes is shown in Figure S4.

Discussion

The present study has identified SNPs located upstream or within the *ATP2B1* gene as strong susceptibility polymorphisms for hypertension in Japanese. These are findings that have also been reported recently in individuals of European descent[3] and in Koreans.[4] Although numerous studies have attempted to identify genetic markers for hypertension over the past 2 decades, there has been little cross-validation of loci in different ethnic groups so far except for mendelian forms of hypertension. The SNPs in *ATP2B1* identified in this study showed significant association in large-scale studies in populations with different ancestries and using different discovery approaches, including GWAS in the CHARGE consortium and the Korean study and an independent candidate gene analysis in our present study. Similar findings in different ethnic groups with different methods further strengthen these findings and indicate the *ATP2B1* gene region as a susceptibility locus of likely global significance for BP variation and development of hypertension. Two replication results very recently reported by another Japanese group[12] and a Korean group[13] also indicated the disease susceptibility of *ATP2B1* SNPs located in the same LD block.

No biological data have been provided whether SNP rs1105378 or other SNPs in strong LD have any effect on the transcriptional activity or transcriptional regulation of the *ATP2B1* gene. Furthermore, although alternative splicing has been found to generate several variants of *ATP2B1* mRNA,[14] the SNP associations that we have observed do not shed light on whether this is a potential mechanism for affecting BP. Our data first showed that the effect of SNPs on ATP2B1 gene expression levels is a potential mechanism by which disease-associated SNP alleles cause the phenotypic changes. Changes in the *ATP2B1* gene product levels are involved in BP regulation. We found no microRNA harboring rs11105378 in the miRBase database.[15]

The *ATP2B1* (so-called *PMAC1*) gene encodes the plasma membrane calcium ATPase isoform 1, which removes bivalent calcium ions from eukaryotic cells against very large concentration gradients and plays a critical role in intracellular calcium homeostasis. Although pathophysiological implications of *ATP2B1* gene products on the development of hypertension are uncertain, it has been reported that inhibition of *ATP2B1* by the selective inhibitor caloxin 2A1 showed endothelium-dependent relaxation of rat aorta by increasing cytosolic Ca^{2+} concentration and consequent activation of endothelial NO synthase.[16] Other information on the role of *ATP2B1* has been obtained from experiments using bladder smooth muscle cells: contractility measurements on these cells have documented the important role of *ATP2B1* in the extrusion of Ca^{2+} after carbachol stimulation or depolarization with potassium chloride.[17] These reports suggest altered vascular reactivity as a plausible explanation for disease susceptibility of *ATP2B1* gene.

In mammals, calcium ATPase isoforms are encoded by ≥4 separate genes (*ATP2B1* to *ATP2B4*).[18] It has been reported that overexpression of the human *ATP2B4* gene in arterial smooth muscle cells in mice increases vascular reactivity and BP partly because of negative regulation of neuronal NO synthase.[19] We, therefore, examined the possible association of *ATP2B4* gene polymorphisms with hypertension by using the screening panel. However, no significant correlation was observed in the 17 SNPs analyzed, which were selected by reference to the HapMap database. The pathophysiological association of plasma membrane Ca^{2+} pump with BP regulation may be isoform specific.

Numerous studies, including the recent GWAS,[3-6] have attempted to identify genetic variations associated with human BP levels. At present, it is not clear to what extent findings from GWAS in one population can be extrapolated

to other populations with different lifestyles and genetic background. However, the present study provides a cross-validation of 4 of 7 SNPs (most likely representing 3 of 6 independent signals) derived from European GWAS. Replication studies in other Japanese[12] and Korean[13] populations also reported the cross-validation of European GWAS-derived SNP. Conservation of susceptible loci for hypertension was independent of ethnic background. This finding suggests an existence of unidentified common etiology of essential hypertension in relation to the susceptible genes and their physiological pathways.

Although individual common genetic variants confer a modest risk of hypertension, their combination showed a large impact on hypertension. The genetic risk score was associated with ≤2.27-times greater odds for hypertension. Similar observations have been found in other common diseases and multifactorial phenotypes, including, for example, type 2 diabetes mellitus,[20] serum lipid levels,[21] and serum uric acid levels.[22] We reported previously that the findings of the cross-sectional analysis revealed a similar association in the longitudinal analysis[23]; the fat mass and obesity-associated gene polymorphism was an independent risk factor for the future development of obesity after adjustment for possible confounding factors. The present cross-sectional study cannot address the question of whether the *ATP2B1* polymorphism and other susceptible variants predict future development of hypertension. However, recent articles investigating a prognostic significance of susceptible variants for type 2 diabetes mellitus[24] and cardiovascular disease[25] showed poor predictive performance of common variants in spite of the high OR observed in subjects carrying multiple risk alleles. A small proportion of the genetically high-risk persons attributed to independent inheritance of risk alleles may make it difficult to discriminate intermediate-risk persons. Genetic information may be most useful to identify a high-risk individual's need for early intervention.

Several definitions of hypertension were used in this study to explore susceptible SNPs with modest effects and to further validate the susceptibility. Since it was expected to be underpowered to detect the effects of common variants in a dichotomized analysis with slightly elevated BP, subjects with high normal BP were excluded from the 65 347 case-control analyses. All of the alleles associated with hypertension in a dichotomized analysis (Table S7) were also associated with BP levels (Table 2). Our methodology may, thus, be appropriate to identify susceptible variants for hypertension.

Perspectives

We have identified SNPs located in the *ATP2B1* gene region as susceptibility loci for hypertension in Japanese using a multistage association study, an association that has now been confirmed across different ethnic groups. Differences in the ex vivo *ATP2B1* mRNA expression levels further supported the disease susceptibility of SNP rs1110578. We also replicated the susceptibility of the European GWAS-derived SNPs in Japanese. Because hypertension is a trait that is preventable by dietary and exercise interventions, early detection of at-risk populations using genetic information may be useful in preventing future hypertension-related diseases.

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Disclosures

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ONLINE SUPPLEMENT

Common variants in the ATP2B1 gene are associated with susceptibility to hypertension
The Japanese Millennium Genome Project

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SUPPLEMENTAL METHODS

ex vivo expression analysis of ATP2B1 mRNA

We obtained 34 umbilical cords at delivery (Kosei General Hospital). Umbilical arteries were excised from the cords and cut into small pieces. Umbilical artery smooth muscle cells (UASMCs) were separated using Hanks buffer containing 2 mg/ml collagenase and cultured in HuMedia-SG (Kurabo, Osaka, Japan) supplemented with epithelial growth factor (0.5 ng/ml), basic fibroblast growth factor (2 ng/ml), insulin (5 μg/ml), antibiotics and 5% fetal bovine serum. Total RNAs was extracted from UASMCs during early passages using TRIzol reagent according to manufacturer's instructions (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized from 500 ng of the total RNA using a PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan), and then diluted five times for subsequent real-time PCR (RT-PCR). RT-PCR was performed using TaqMan Gene Expression Assays on a 7900HT Sequence Detection System (Applied Biosystems). A relative quantification method [1] was used to measure the amounts of ATP2B1 (TaqMan assay ID, Hs00155949_m1) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1) as an internal control. Genotype of ATP2B1 rs11105378 of each sample was analyzed by direct sequencing (BigDye Terminator v3.1 Cycle Sequencing Kit on a 3730xl GeneticAnalyzer, Applied Biosystems) using isolated genomic DNA from UASMCs (QIAamp DNA Mini Kit, QIAGEN GmbH, Hilden, Germany). The direct sequencing was performed with the following primers; forward 5'-TTCATAGCCCTTTTCATCTCTTTC-3', reverse 5'-AGAATCTCGGGAAAACAGCA-3'.

Table S1 Clinical characteristics of the cohort-based population sample

Parameters	Total (14,105)	Community-based general population					Company employee	
		Ohasama (1,592)	Shigaraki (2,273)	Takashima (1,730)	Suita (2,536)	Nomura (2,876)	Yokohama (2,290)	Matsuyama (808)
Age (years)	57.8±14.0	57.5±11.2	57.2±15.5	59.7±14.1	65.6±10.9	61.1±14.0	45.7±10.2	54.2±5.8
Sex (male/female)	6931/7174	601/991	862/1411	633/1097	1160/1376	1247/1629	1659/631	769/39
Body mass index (kg/m^2)	23.0±3.1	23.7±3.2	22.6±3.1	22.9±3.0	22.9±3.1	23.4±3.2	22.4±3.1	23.4±2.9
History of CVD	7.1	11.9	12.1	4.0	7.5	8.1	0.4	4.3
Systolic BP (mmHg)	130.1±19.6	131.7±14.2	130.1±19.5	130.6±21.3	124.5±18.9	137.7±22.1	123.8±14.9	134.3±19.1
Diastolic BP (mmHg)	77.9±11.5	74.4±9.4	76.7±11.7	76.8±12.0	75.6±10.5	81.0±11.8	78.3±10.3	85.1±12.2
Hypertension (%)	40.7	43.2	44.4	39.5	38.2	53.3	22.9	46.2
Antihypertensive treatment (%)	20.5	26.5	23.5	16.4	26.4	25.7	6.5	12.4

Values are mean±SD. Cardiovascular disease (CVD); stroke, myocardial infarction, and angina pectoris. Hypertension; any or all of systolic blood pressure more than 140 mmHg, diastolic blood pressure more than 90 mmHg, and current use of antihypertensive agents. The Ohasama study conducted by Tohoku University is a population-based longitudinal epidemiological study focusing on the clinical implications of home BP measurement [2]. Ohasama Town is a rural community located in the northern part of Japan (Iwate Prefecture). Subjects were recruited through a community-based annual medical check-up process. The Shigaraki [3] and Takashima [4]| studies of Shiga University of Medical Science are general population-based longitudinal studies. Both towns are located in central Japan (Shiga Prefecture). Subjects were recruited through a community-based annual medical check-up process. The Suita study conducted by the National Cardiovascular Center is based on the residents of Suita city, an urban city located in the second largest area Osaka, Japan [5]. Subjects were recruited through a biennial medical check-up process of the National Cardiovascular Center. The Nomura study of Ehime University is a longitudinal epidemiological study based on the Nomura Town residents, a largely rural community located in Ehime Prefecture [6]. Subjects were recruited through a community-based annual medical check-up process. The Yokohama (Yokohama City University) and Matsuyama (Ehime University) cohorts are derived from employees of large manufacturing industries located in Kanagawa and Matuyama City, Ehime Prefecture (western part of Japan) [7] respectively. In all cohorts, clinical parameters were obtained from personal health records during the annual or biennial medical check-up process. All study procedures were approved by the ethics committee of each University or Institution. Singed informed consent was obtained from all participating subjects.

Table S2 Clinical characteristics of the replication panel

Parameters	Hypertensive cases (1,929)	Normotensive controls (1,993)	p
Age (years)	55.1±7.1	55.2±9.5	0.680
Sex (male/female)	1,200/729	829/1,164	<0.001
Body mass index (kg/m^2)	24.4±3.1	21.9±2.7	<0.001
History of CVD (%)	5.4	0	<0.001
Systolic blood pressure (mmHg)	146.3±15.9	109.5±7.5	<0.001
Diastolic blood pressure (mmHg)	91.0±10.1	67.7±6.5	<0.001
Antihypertensive treatment (%)	47.5	0	<0.001

Values are mean±SD. Nested hypertensive cases and normotensive control subjects were chosen from the cohort-based population sample according to the following criteria: hypertensive subjects aged 64 years or younger, and were either being treated with antihypertensive medication or had a SBP more than 160 mmHg and/or DBP more than 90 mmHg; normotensive subjects aged 40 years or older, and all of SBP less than 120 mmHg, and DBP less than 80 mmHg, no current use of antihypertensive medication, and free from any history of cardiovascular disease. Cardiovascular disease (CVD) includes stroke, myocardial infarction, and angina pectoris.

Table S3 Association of 36 candidate SNPs with hypertension (replication panel)

Gene	SNP (position)	Genotype		Screening Panel					Odds ratio (p-value)			
				Genotype frequency			HWE	Call rate	Allelic	Recessive	Dominant	Additive
ACCN1	rs28933	AA/GA/GG	HT	464	974	449	0.159	97.6	1.03 (0.479)	1.07 (0.385)	1.02 (0.766)	(0.686)
			NT	469	986	485	0.466					
ADORA1	rs3766554	AA/GA/GG	HT	424	923	557	0.262	98.6	1.03 (0.548)	1.00 (0.977)	1.09 (0.289)	(0.523)
			NT	410	981	574	0.808					
ATP10A	rs3736186	GG/AG/AA	HT	791	868	263	0.312	99.4	**1.10 (0.040)**	1.04 (0.666)	**1.18 (0.010)**	**(0.033)**
			NT	734	963	280	0.206					
ATP10D	rs1058793	AA/GA/GG	HT	675	894	325	0.326	98.2	1.07 (0.147)	1.17 (0.060)	1.04 (0.555)	(0.169)
			NT	680	896	382	0.005					
ATP2A3	rs887387	TT/TC/CC	HT	936	775	189	0.126	98.7	1.05 (0.342)	1.02 (0.840)	1.07 (0.263)	(0.527)
			NT	936	836	200	0.508					
ATP2B1	rs2070759	GG/GT/TT	HT	582	896	399	0.118	97.2	**1.18 ($4.0*10^{-4}$)**	**1.2 (0.018)**	**1.27 (0.001)**	**(0.002)**
			NT	507	956	474	0.579					
CACNA1E	rs2293990	AA/TA/TT	HT	568	911	412	0.194	98.2	1.03 (0.532)	1.07 (0.372)	1.01 (0.881)	(0.661)
			NT	585	926	451	0.022					
CACNA2D2	rs2236957	GG/GA/AA	HT	459	925	496	0.499	97.3	1.00 (0.948)	1.00 (0.972)	1.01 (0.943)	(0.997)
			NT	471	954	512	0.523					
EAST	rs967591	AA/AG/GG	HT	442	916	552	0.100	99.1	1.00 (0.932)	0.98 (0.725)	1.02 (0.814)	(0.875)
			NT	451	964	561	0.345					
CHGA	rs3759717	CC/TC/TT	HT	744	877	288	0.263	99.1	1.00 (0.977)	0.93 (0.434)	1.04 (0.598)	(0.522)
			NT	755	943	281	0.624					
COL4A1	rs2305080	GG/GA/AA	HT	485	908	523	0.023	99.2	1.02 (0.723)	0.97 (0.707)	1.07 (0.332)	(0.468)
			NT	473	972	528	0.536					

Table S3 Continued

Gene	SNP (position)	Genotype		Screening Panel					Odds ratio (p-value)			
				Genotype frequency			HWE	Call rate	Allelic	Recessive	Dominant	Additive
DLGAP2	rs2301963	CC/CA/AA	HT	510	904	493	0.024	98.6	1.05 (0.239)	1.07 (0.368)	1.08 (0.321)	(0.516)
			NT	497	932	532	0.029					
ERCC1	rs2298881	CC/CA/AA	HT	595	899	387	0.161	97.5	1.00 (0.948)	0.96 (0.642)	1.04 (0.616)	(0.702)
			NT	600	955	388	0.821					
EXOSC3	rs7158	AA/AG/GG	HT	511	967	418	0.327	97.9	1.01 (0.850)	1.09 (0.264)	0.95 (0.452)	(0.262)
			NT	545	941	458	0.187					
FGF2	rs3747676	GG/GA/AA	HT	415	937	519	0.839	96.4	1.01 (0.892)	1.07 (0.340)	0.94 (0.424)	(0.309)
			NT	444	908	556	0.050					
GIPC1	rs3815715	GG/GA/AA	HT	734	863	309	0.040	98.8	1.03 (0.585)	0.98 (0.794)	1.07 (0.330)	(0.510)
			NT	728	927	313	0.532					
GNA14	rs1801258	TT/TC/CC	HT	317	919	675	0.888	99.0	1.05 (0.321)	1.11 (0.128)	0.90 (0.903)	(0.249)
			NT	330	899	743	0.039					
GNAI2	rs2236943	GG/GA/AA	HT	556	912	429	0.137	97.9	1.04 (0.427)	1.02 (0.751)	1.07 (0.345)	(0.640)
			NT	543	953	448	0.448					
GUCA1C	rs2715709	AA/GA/GG	HT	225	886	767	0.204	97.1	1.06 (0.242)	1.12 (0.081)	0.98 (0.824)	(0.156)
			NT	236	853	843	0.373					
HCN4	rs3743496	GG/TG/TT	HT	431	877	594	0.002	98.2	1.01 (0.859)	0.94 (0.369)	1.11 (0.192)	(0.150)
			NT	408	959	583	0.710					
HLA-DMB	rs2071556	CC/CA/AA	HT	511	932	450	0.534	98.0	1.09 (0.060)	**1.17 (0.035)**	1.07 (0.346)	(0.105)
			NT	500	928	521	0.036					
KCNIP2	rs755381	TT/TC/CC	HT	453	904	543	0.044	98.2	1.05 (0.245)	1.03 (0.688)	1.12 (0.128)	(0.311)
			NT	425	957	569	0.548					

Table S3 Continued

Gene	SNP (position)	Genotype		Screening Panel					Odds ratio (p-value)			
				Genotype frequency			HWE	Call rate	Allelic	Recessive	Dominant	Additive
KCNMB4	rs710652	CC/AC/AA	HT	660	953	298	0.131	99.2	1.09 (0.056)	**1.28 (0.003)**	1.03 (0.638)	**(0.012)**
			NT	669	930	379	0.083					
KCNN1	rs2278993	TT/TC/CC	HT	189	805	919	0.513	99.2	1.07 (0.152)	1.08 (0.259)	1.15 (0.207)	(0.335)
			NT	172	819	985	0.924					
PPP1R1B	rs3764352	TT/TC/CC	HT	547	940	374	0.412	96.0	1.07 (0.156)	1.16 (0.059)	1.04 (0.621)	(0.165)
			NT	546	928	431	0.333					
PTHR1	rs1138518	TT/TC/CC	HT	381	931	595	0.626	98.5	1.01 (0.814)	1.04 (0.599)	0.98 (0.843)	(0.803)
			NT	396	935	626	0.169					
PTPRT	rs3746539	AA/AG/GG	HT	495	991	430	0.119	99.1	1.04 (0.435)	1.12 (0.139)	0.99 (0.863)	(0.262)
			NT	514	975	482	0.644					
RAC2	rs929023	TT/TC/CC	HT	387	921	588	0.448	98.2	1.06 (0.200)	1.06 (0.438)	1.12 (0.173)	(0.373)
			NT	365	961	629	0.951					
RGS2	rs3767489	AA/GA/GG	HT	635	892	370	0.075	98.0	1.03 (0.483)	0.94 (0.476)	1.12 (0.099)	(0.104)
			NT	603	981	362	0.291					
RGS20	rs3816772	CC/CG/GG	HT	268	924	695	0.162	97.6	1.03 (0.543)	1.11 (0.132)	0.92 (0.377)	(0.112)
			NT	295	884	760	0.152					
SLC13A1	rs2140516	GG/GA/AA	HT	341	917	662	0.448	99.4	1.06 (0.225)	1.11 (0.135)	1.03 (0.736)	(0.322)
			NT	343	907	727	0.039					
SLC22A7	rs2270860	AA/GA/GG	HT	233	868	788	0.800	97.8	**1.1 (0.048)**	**1.15 (0.032)**	1.09 (0.406)	(0.100)
			NT	223	844	878	0.352					
SLC26A8	rs2295852	TT/TC/CC	HT	994	747	154	0.413	97.6	1.01 (0.835)	0.97 (0.806)	1.03 (0.690)	(0.857)
			NT	1002	779	153	0.926					

Table S3 Continued

Gene	SNP (position)	Genotype		Screening Panel					Odds ratio (p-value)			
				Genotype frequency			HWE	Call rate	Allelic	Recessive	Dominant	Additive
SLC2A11	rs2236620	AA/AG/GG	HT	308	890	715	0.266	99.0	1.04 (0.360)	1.00 (0.956)	1.16 (0.092)	(0.211)
			NT	279	953	738	0.306					
SLCO1B1	rs2291075	GG/GA/AA	HT	719	868	319	0.039	98.7	1.01 (0.866)	0.95 (0.524)	1.05 (0.466)	(0.493)
			NT	719	932	314	0.680					
WNK1	rs2255390	GG/GA/AA	HT	490	925	475	0.359	97.4	1.07 (0.139)	1.09 (0.262)	1.10 (0.201)	(0.339)
			NT	466	949	516	0.470					

The replication panel consists of 1,929 hypertensive cases and 1,993 normotensives controls selected from a 11,569 cohort sample (Table S2).

Table S4 Clinical characteristics of the screening panel

Parameters	Hypertensive cases (758)	Normotensive controls (726)
Male (n (%))	564 (74.4)	550 (75.8)
Age (years)	59.0±11.0	62.8±9.4
Body mass index (kg/m^2)	23.6±3.0	22.7±2.9
Systolic BP (mmHg)	163.5±24.6	115.9±12.0
Diastolic BP (mmHg)	100.3±15.7	72.0±7.6
Antihypertensive medication (n (%))	499 (65.8)	-

Values are mean±standard deviation. Hypertensive cases: non-obese hypertensive patients, who had a previous diagnosis of hypertension at between 30 and 59 years of age, were either being treated with antihypertensive medication or had a SBP more than 160 mmHg and/or DBP more than 100 mmHg, had a family history of hypertension in their parents and/or siblings. Normotensive controls: middle-aged to elderly subjects (aged more than 45 years), who had never been treated with antihypertensive medications, had a SBP less than 120 mmHg and DBP less than 80 mmHg, and had no family history of hypertension.

Table S5 Dense SNP analysis of the *ATP2B1* gene (screening panel)

Gene	SNP (position)	Genotype		Screening Panel					Odds ratio (p-value)			
				Genotype frequency			HWE	Call rate	Allelic	Recessive	Dominant	Additive
ATP2B1	rs3920010 (88464519)	GG/GA/AA	HT	17	191	542	0.971	97.9	0.95 (0.591)	0.72 (0.311)	0.97 (0.808)	(0.596)
			NT	22	177	504	0.187					
	rs3900133 (88512561)	CC/CA/AA	HT					NF				
			NT									
	rs1401982 (88513730)	AA/AG/GG	HT	318	328	92	0.603	96.3	1.28 (0.001)	1.34 (0.007)	1.45 (0.014)	(0.006)
			NT	249	324	118	0.474					
	rs988111 (88515650)	TT/TC/CC	HT					NF				
			NT									
	rs10858912 (88515998)	GG/GA/AA	HT					NF				
			NT									
	rs4516026 (88518251)	TT/TG/GG	HT					NF				
			NT									
	rs2854371 (88519597)	GG/GA/AA	HT	23	208	520	0.692	98.7	1.32 (0.008)	1.38 (0.333)	1.37 (0.008)	(0.028)
			NT	16	159	538	0.300					
	rs1520184 (88520698)	GG/GA/AA	HT					NF				
			NT									
	rs1356819 (88524892)	AA/AC/CC	HT	743	5	0	0.927	98.6	1.26 (0.707)	1.26 (0.706)		
			NT	709	6	0	0.910					
	rs957525 (88524946)	TT/TC/CC	HT	414	264	62	0.034	97.6	1.05 (0.554)	1.11 (0.303)	0.90 (0.599)	(0.389)
			NT	377	277	54	0.753					
	rs17017109 (88528238)	TT/TG/GG	HT	591	144	7	0.586	97.8	0.81 (0.094)	0.79 (0.079)	0.89 (0.842)	(0.211)
			NT	591	113	6	0.816					

Table S5 Continued

Gene	SNP (position)	Genotype		Screening Panel					Odds ratio (p-value)			
				Genotype frequency			HWE	Call rate	Allelic	Recessive	Dominant	Additive
ATP2B1	rs1520183 (88532742)	CC/CT/TT	HT					NF				
			NT									
	rs2681472 (88533090)	GG/GA/AA	HT	90	321	335	0.334	97.8	0.79 (0.003)	0.74 (0.044)	0.75 (0.006)	(0.012)
			NT	111	328	267	0.539					
	rs11614886 (88535251)	GG/GC/CC	HT					NF				
			NT									
	rs2070759 (88541867)	GG/GT/TT	HT	216	379	151	0.515	97.6	1.15 (0.054)	1.13 (0.297)	1.31 (0.033)	(0.096)
			NT	186	341	175	0.454					
	rs2070758 (88545352)	AA/AC/CC	HT	638	103	10	0.016	98.4	1.23 (0.113)	1.32 (0.050)	0.63 (0.377)	(0.056)
			NT	575	128	6	0.701					
	rs1050395 (88553032)	TT/TC/CC	HT	730	17	0	0.753	97.9	1.38 (0.327)	1.32 (0.406)		(0.468)
			NT	685	20	1	0.042					
	rs1050396 (88553110)	CC/CA/AA	HT					NF				
			NT									
	rs2056327 (88562685)	CC/CT/TT	HT					NF				
			NT									
	rs939329 (88564015)	AA/AG/GG	HT	196	382	168	0.485	97.4	1.08 (0.313)	1.04 (0.726)	1.18 (0.190)	(0.422)
			NT	178	343	178	0.623					
	rs7975689 (88571125)	AA/AG/GG	HT					NF				
			NT									
	rs7138016 (88572551)	TT/TA/AA	HT					NF				
			NT									

Table S5 Continued

Gene	SNP (position)	Genotype		Screening Panel					Odds ratio (p-value)			
				Genotype frequency			HWE	Call rate	Allelic	Recessive	Dominant	Additive
ATP2B1	rs12579302 (88574634)	GG/GA/AA	HT	105	310	333	0.018	98.9	0.80 (0.004)	0.76 (0.058)	0.76 (0.011)	(0.023)
			NT	127	319	273	0.046					
	rs11105359 (88575212)	TT/TG/GG	HT					NF				
			NT									
	rs11105360 (88575303)	TT/TC/CC	HT					NF				
			NT									
	rs11105361 (88576810)	CC/CA/AA	HT					NF				
			NT									
	rs7131965 (88590466)	TT/TC/CC	HT	731	15	0	0.025	98.7	0.90 (0.778)	0.83 (0.627)		(0.468)
			NT	707	11	1	0.990					
	rs11105364 (88593407)	TT/TG/GG	HT	335	322	88	0.276	97.2	1.29 (0.001)	1.36 (0.004)	1.44 (0.016)	(0.005)
			NT	261	323	113	0.295					
	rs11105368 (88598572)	GG/GC/CC	HT	349	284	89	0.883	94.0	1.25 (0.005)	1.21 (0.082)	1.53 (0.005)	(0.015)
			NT	294	260	119	0.212					
	rs7136259 (88605319)	TT/TC/CC	HT	323	325	87	0.348	97.2	1.24 (0.006)	1.22 (0.063)	1.50 (0.007)	(0.016)
			NT	277	312	119	0.389					
	rs17836871 (88606297)	TT/TC/CC	HT	419	260	61	0.025	97.8	1.08 (0.368)	1.16 (0.153)	0.90 (0.577)	(0.202)
			NT	376	282	53	0.990					
	rs11105378 (88614872)	TT/TC/CC	HT	76	301	359	0.276	97.3	0.73 ($6.3*10^{-5}$)	0.64 (0.005)	0.69 ($4.2*10^{-4}$)	($4.6*10^{-4}$)
			NT	108	320	280	0.295					
	rs12230074 (88614998)	GG/GA/AA	HT	83	328	332	0.883	97.6	0.82 (0.013)	0.70 (0.021)	0.82 (0.068)	(0.036)
			NT	108	316	282	0.212					

Table S5 Continued

Gene	SNP (position)	Genotype		Screening Panel					Odds ratio (p-value)			
				Genotype frequency			HWE	Call rate	Allelic	Recessive	Dominant	Additive
ATP2B1	rs11105379 (88619304)	TT/TC/CC	HT	450	240	39	0.348	96.3	1.11 (0.261)	1.12 (0.292)	1.16 (0.520)	(0.542)
			NT	413	244	43	0.389					
	rs10858918 (88620476)	TT/TC/CC	HT	40	266	442	0.998	98.6	0.90 (0.212)	0.82 (0.378)	0.89 (0.267)	(0.456)
			NT	46	267	402	0.852					
	rs2113894 (88623528)	AA/AT/TT	HT	459	232	43	0.063	96.3	1.12 (0.200)	1.14 (0.228)	1.17 (0.482)	(0.458)
			NT	413	235	47	0.090					
	rs1358350 (88626023)	TT/TA/AA	HT	49	202	445	<0.001	91.8	0.85 (0.085)	0.82 (0.345)	0.84 (0.113)	(0.263)
			NT	56	212	398	<0.001					
	rs12369944 (88626925)	CC/CA/AA	HT	617	97	15	<0.001	94.5	1.27 (0.066)	1.33 (0.043)	1.01 (0.976)	(0.104)
			NT	542	117	14	0.013					
	rs2280715 (88627833)	CC/CG/GG	HT	463	223	54	<0.001	97.0	1.14 (0.137)	1.16 (0.166)	1.17 (0.425)	(0.364)
			NT	413	228	59	0.001					
	rs11105381 (88630966)	GG/GA/AA	HT	452	259	37	0.990	98.2	1.09 (0.334)	1.09 (0.398)	1.18 (0.479)	(0.621)
			NT	413	255	41	0.843					
	rs1590008 (88631856)	TT/TC/CC	HT	438	265	42	0.818	98.2	1.11 (0.243)	1.12 (0.288)	1.18 (0.443)	(0.508)
			NT	399	266	47	0.767					

The screening panel is comprised of 758 middle age-onset severe hypertensive patients and 726 middle-aged to elderly evidently normotensive controls (Table S4). NF; no genotype frequency

Table S6 Association of 17 ATP2B4 SNPs with hypertension (screening panel)

Gene	SNP (position)	Genotype		Screening Panel					Odds ratio (p-value)			
				Genotype frequency			HWE	Call rate	Allelic	Recessive	Dominant	Additive
ATP2B4	rs4245719	GG/GA/AA	HT	287	343	117	0.389	98.5	0.90 (0.153)	0.90 (0.307)	0.82 (0.175)	(0.332)
			NT	293	327	94	0.854					
	rs4600103	GG/GA/AA	HT	286	312	129	0.007	94.3	1.03 (0.678)	1.08 (0.466)	0.98 (0.860)	(0.685)
			NT	252	304	117	0.128					
	rs17537593	TT/TA/AA	HT	64	237	432	<0.001	96.6	1.03 (0.704)	1.33 (0.154)	0.97 (0.761)	(0.252)
			NT	47	246	407	0.240					
	rs4951273	GG/GC/CC	HT	114	339	289	0.377	97.9	1.11 (0.178)	1.21 (0.214)	1.11 (0.323)	(0.383)
			NT	93	323	295	0.756					
	rs12749310	GG/GA/AA	HT	427	245	56	0.014	96.1	1.03 (0.766)	1.10 (0.370)	0.81 (0.305)	(0.256)
			NT	393	261	44	0.940					
	rs4297354	GG/GA/AA	HT	462	227	40	0.087	96.1	**1.20 (0.047)**	**1.27 (0.028)**	1.11 (0.662)	(0.086)
			NT	402	253	42	0.794					
	rs11576343	TT/TC/CC	HT	53	251	432	0.051	97.3	0.92 (0.323)	1.02 (0.918)	0.87 (0.202)	(0.382)
			NT	50	266	392	0.597					
	rs6594013	TT/TA/AA	HT	163	348	231	0.141	97.9	0.95 (0.443)	0.98 (0.856)	0.89 (0.310)	(0.587)
			NT	159	348	204	0.647					
	rs16852152	GG/GA/AA	HT	437	252	38	0.831	95.9	0.92 (0.354)	0.92 (0.449)	0.82 (0.418)	(0.618)
			NT	432	234	30	0.812					
	rs3766752	GG/GA/AA	HT	210	367	167	0.782	97.8	1.09 (0.225)	1.15 (0.235)	1.10 (0.433)	(0.454)
			NT	180	356	171	0.847					
	rs11808688	GG/GA/AA	HT	197	372	169	0.795	96.9	0.94 (0.389)	0.86 (0.183)	1.00 (0.985)	(0.370)
			NT	209	331	160	0.189					

Table S6 Continued

Gene	SNP (position)	Genotype		Screening Panel					Odds ratio (p-value)			
				Genotype frequency			HWE	Call rate	Allelic	Recessive	Dominant	Additive
ATP2B4	rs4951130	GG/GA/AA	HT	410	278	50	0.758	97.2	**1.21 (0.025)**	1.22 (0.058)	1.40 (0.086)	(0.082)
			NT	356	283	65	0.421					
	rs12095268	TT/TA/AA	HT	367	313	67	0.982	98.0	1.09 (0.303)	1.09 (0.439)	1.19 (0.335)	(0.556)
			NT	333	300	74	0.599					
	rs12410036	TT/TC/CC	HT	48	256	439	0.200	97.7	0.90 (0.232)	0.93 (0.720)	0.87 (0.196)	(0.434)
			NT	49	264	394	0.599					
	rs7547344	GG/GA/AA	HT	172	362	205	0.618	97.7	1.00 (0.977)	1.02 (0.875)	0.98 (0.846)	(0.954)
			NT	163	354	194	0.951					
	rs955865	GG/GA/AA	HT	208	368	173	0.677	98.6	0.95 (0.456)	0.96 (0.733)	0.89 (0.370)	(0.668)
			NT	204	359	151	0.765					
	rs955866	TT/TC/CC	HT	170	366	208	0.712	98.5	1.05 (0.489)	1.11 (0.401)	1.04 (0.756)	(0.702)
			NT	151	361	206	0.758					

The screening panel is comprised of 758 middle age-onset severe hypertensive patients and 726 middle-aged to elderly evidently normotensive controls (Table S4).

Table S7 Meta-analysis of ATP2B1 SNPs with hypertension

SNP	Coded Allele	Millennium GPJ			Global BPgen			Pooled		
		OR (95% CI)	P	N	OR (95% CI)	P	N	OR (95% CI)	P	N
rs1401982	A	1.19 (1.11-1.29)	$1.3*10^{-6}$	9,967	1.07 (1.02-1.12)	0.010	19126	1.10 (1.06-1.15)	$1.5*10^{-6}$	29,093
rs2681472	A	1.21 (1.13-1.30)	$1.8*10^{-7}$	10,039	1.14 (1.06-1.22)	$2.2*10^{-4}$	19055	1.17 (1.12-1.23)	$2.1*10^{-10}$	29,094
rs11105364	T	1.21 (1.13-1.30)	$1.5*10^{-7}$	10,014	1.13 (1.06-1.21)	$4.6*10^{-4}$	19151	1.17 (1.11-1.22)	$3.1*10^{-10}$	29,165
rs11105378	C	1.21 (1.13-1.30)	$1.5*10^{-7}$	9,972	1.13 (1.05-1.21)	$5.9*10^{-4}$	18894	1.17 (1.11-1.23)	$7.0*10^{-10}$	28,866

In both Japanese Millennium GPJ and Global BP gen, hypertensive subjects were defined as being treated with antihypertensive medication, or SBP greater or equal to 140 mmHg, or DBP greater or equal to 90 mmHg; normotensive subjects were defined as all of not treated with antihypertensive medication, and SBP less or equal to 120 mmHg, and DBP less or equal to 85 mmHg [8]. Adjusted odds ratio was calculated under additive model using multiple logistic regression analysis adjusted for age, age^2, sex, BMI, and cohort variables. Within Global BPgen, individual cohort results were combined using inverse variance weighted meta-analysis of the effects on a log-odds-ratio scale.

Table S8 Association of ATP2B1 SNPs and blood pressure traits in each Japanese cohort

SNP	coded allele		cohort				SBP			DBP		
	allele	%	name	n	HWE	CR	coefficient	SE	p	coefficient	SE	p
rs1401982	A	61.9	Ohasama	1569	0.227	98.6	0.35	0.60	0.558	0.06	0.39	0.868
		62.3	Yokohama	2269	0.588	99.1	-1.51	0.43	**$4.2*10^{-4}$**	-0.75	0.29	**0.009**
		62.6	Shigaraki	2191	0.908	96.4	-1.72	0.56	**0.002**	-0.91	0.35	**0.010**
		61.8	Takashima	1718	0.302	99.3	-1.95	0.72	**0.007**	-0.90	0.41	**0.028**
		61.7	Suita	2529	0.506	99.7	-0.80	0.57	0.160	-0.44	0.33	0.182
		62.0	Matsuyama	803	0.175	99.4	-1.27	0.97	0.194	-1.39	0.62	**0.026**
		63.8	Nomura	2865	0.611	99.6	-1.39	0.56	**0.020**	-0.67	0.33	**0.045**
rs2681472	A	62.1	Ohasama	1587	0.226	99.7	0.38	0.60	0.522	0.06	0.39	0.887
		62.6	Yokohama	2278	0.321	99.5	-1.52	0.43	**$3.8*10^{-4}$**	-0.78	0.28	**0.006**
		63.5	Shigaraki	2254	0.701	99.2	-2.03	0.56	**$2.9*10^{-4}$**	-1.15	0.35	**0.001**
		62.3	Takashima	1718	0.257	99.3	-2.25	0.72	**0.002**	-1.03	0.41	**0.013**
		62.1	Suita	2528	0.655	99.7	-0.97	0.57	0.089	-0.49	0.33	0.131
		62.1	Matsuyama	802	0.191	99.3	-1.13	0.98	0.248	-1.39	0.62	**0.026**
		64.3	Nomura	2865	0.907	99.6	-1.42	0.60	**0.018**	-0.69	0.34	**0.041**
rs11105364	T	62.2	Ohasama	1589	0.203	99.8	0.42	0.60	**0.477**	0.12	0.39	**0.766**
		63.3	Yokohama	2277	0.414	99.4	-1.61	0.43	**$1.8*10^{-4}$**	-0.79	0.29	**0.006**
		64.3	Shigaraki	2234	0.410	98.3	-2.11	0.56	**$1.7*10^{-4}$**	-1.16	0.35	**0.001**
		62.7	Takashima	1727	0.570	99.8	-2.25	0.71	**0.002**	-0.98	0.41	**0.017**
		62.4	Suita	2530	0.635	99.8	-1.08	0.57	**0.058**	-0.54	0.33	**0.096**
		62.8	Matsuyama	805	0.285	99.6	-1.05	0.98	**0.285**	-1.35	0.62	**0.031**
		64.4	Nomura	2851	0.495	99.1	-1.30	0.60	**0.030**	-0.60	0.34	**0.077**

Table S8 Continued

SNP	coded allele		cohort				SBP			DBP		
	allele	%	name	n	HWE	CR	coefficient	SE	p	coefficient	SE	p
		62.9	Ohasama	1566	0.478	98.4	0.31	0.60	**0.600**	-0.04	0.39	**0.914**
		63.4	Yokohama	2258	0.244	98.6	-1.32	0.43	**0.002**	-0.66	0.29	**0.022**
		65.2	Shigaraki	2213	0.141	97.4	-2.45	0.56	**$1.3*10^{-5}$**	-1.31	0.35	**$2.2*10^{-4}$**
rs11105378	C	63.2	Takashima	1722	0.237	99.5	-2.41	0.72	**$8.5*10^{-4}$**	-1.15	0.41	**0.006**
		63.0	Suita	2521	0.498	99.4	-1.00	0.58	**0.084**	-0.42	0.33	**0.207**
		63.2	Matsuyama	803	0.434	99.4	-1.14	0.99	**0.249**	-1.56	0.63	**0.014**
		65.7	Nomura	2865	0.468	99.6	-1.11	0.60	**0.065**	-0.47	0.34	**0.164**

Coefficients and standardized error for systolic and diastolic BP were calculated under additive model using multiple regression analysis adjusted for age, age2, sex, BMI. Adjustment for treatment with antihypertensive medication was achieved by adding fixed constants to measured values (+15mmHg for SBP and +10mmHg for DBP). CR indicates call rate.

Table S9 Association of European GWAS-derived SNPs with hypertension in the Japanese screening and replication panels

SNP	Genotype		Screening panel						Replication panel						overall
			Genotype frequency			HWE	Call rate	Odds (p value)	Genotype frequency			HWE	Call rate	Odds (p value)	Odds (p value)
FGF5 rs1458038	TT/TC/CC	HT	92	338	315	0.928	98.0	**1.19 0.030**	271	838	788	0.047	97.9	**1.21 ($1.1*10^{-4}$)**	**1.20 ($9.9*10^{-6}$)**
		NT	81	281	347	0.039			225	801	918	0.014			
CYP17A1 rs1004467	AA/AG/GG	HT	380	299	66	0.514	98.6	**1.35 ($1.4*10^{-4}$)**	894	869	168	0.034	99.8	1.09 (0.079)	**1.16 ($4.9*10^{-4}$)**
		NT	309	308	101	0.089			877	901	205	0.236			
CSK rs1378942	CC/CA/AA	HT	483	236	25	0.557	98.0	1.09 0.340	1237	605	72	0.853	98.9	1.04 (0.536)	1.05 (0.305)
		NT	452	223	35	0.274			1259	621	85	0.449			
PLCD3 rs12946454	TT/TA/AA	HT	28	210	510	0.276	98.8	1.12 0.256	68	526	1339	0.070	99.7	0.99 (0.907)	1.03 (0.624)
		NT	13	207	499	0.107			68	545	1364	0.140			
PLEKHA7 rs381815	TT/TC/CC	HT	27	242	483	0.624	98.8	1.05 0.596	85	567	1273	0.033	99.4	0.99 (0.913)	1.01 (0.852)
		NT	31	208	475	0.181			93	574	1308	0.004			
CSK-ULK3 rs6495122	AA/AC/CC	HT	508	204	21	0.924	96.8	1.18 0.085	1289	561	72	0.263	99.2	1.10 (0.102)	**1.12 (0.021)**
		NT	458	221	25	0.793			1267	626	77	0.976			
ULK4 rs9815354	AA/AG/GG	HT	7	142	598	0.654	98.5	0.90 0.374	31	385	1507	0.265	98.9	1.05 (0.463)	1.01 (0.873)
		NT	10	144	561	0.826			26	382	1548	0.659			

The screening panel is comprised of 758 middle age-onset severe hypertensive patients and 726 middle-aged to elderly evidently normotensive controls (Table S4). The replication panel consists of 1,929 hypertensive cases and 1,993 normotensives controls selected from a 11,569 cohort sample were enrolled (Table S2). Odds ratios and p-values for allelic model are shown.

Table S10 Association of European GWAS-derived SNPs and blood pressure traits in each Japanese cohort

SNP	coded allele		cohort				SBP			DBP		
	allele	%	name	n	HWE	CR	coefficient	SE	p	coefficient	SE	p
EGF5 rs1458038	T	33.7	Ohasama	1557	0.174	97.8	1.58	0.60	**0.008**	0.44	0.39	0.260
		33.5	Yokohama	2223	0.005	97.1	0.84	0.44	0.055	0.46	0.29	0.115
		33.8	Shigaraki	2156	0.001	94.9	1.17	0.56	0.037	0.46	0.35	0.196
		31.4	Takashima	1714	0.163	99.1	2.43	0.73	**0.001**	1.62	0.42	**$1.0*10^{-4}$**
		33.6	Suita	2533	0.508	99.9	0.67	0.58	0.250	0.43	0.33	0.191
		33.4	Matsuyama	804	0.459	99.5	0.70	1.04	0.500	0.54	0.67	0.414
		38.2	Nomura	2841	0.105	98.8	1.85	0.58	**0.002**	1.09	0.33	**0.001**
YP17A1 rs1004467	A	70.2	Ohasama	1579	0.254	99.2	1.41	0.45	**0.002**	0.48	0.30	0.110
		68.4	Yokohama	2276	0.812	99.4	1.05	0.57	0.065	0.03	0.36	0.938
		65.5	Shigaraki	2244	0.898	98.7	1.46	0.74	**0.050**	0.83	0.43	0.051
		67.8	Takashima	1714	0.573	99.1	-0.21	0.59	0.721	-0.34	0.34	0.308
		66.8	Suita	2533	0.865	99.9	0.12	1.05	0.911	-0.10	0.67	0.885
		67.4	Matsuyama	804	0.388	99.5	1.25	0.62	0.045	0.50	0.35	0.149
		69.7	Nomura	2859	0.475	99.4	1.41	0.45	**0.002**	0.48	0.30	0.110
SK rs1378942	C	77.7	Ohasama	1575	0.821	98.9	-0.17	0.68	0.804	-0.53	0.45	0.241
		78.1	Yokohama	2245	0.152	98.0	0.73	0.52	0.157	0.48	0.35	0.167
		83.0	Shigaraki	2225	0.187	97.9	1.80	0.71	0.012	1.35	0.45	**0.003**
		80.7	Takashima	1703	0.808	98.4	-0.41	0.88	0.644	0.08	0.51	0.870
		80.5	Suita	2528	0.098	99.7	1.28	0.69	0.063	0.43	0.39	0.270
		79.7	Matsuyama	798	0.846	98.8	0.24	1.21	0.842	0.07	0.77	0.923
		81.0	Nomura	2848	0.075	99.0	1.18	0.72	0.103	0.63	0.41	0.121

Table S10 Continued

SNP	coded allele		cohort				SBP			DBP		
	allele	%	name	n	HWE	CR	coefficient	SE	p	coefficient	SE	p
PLCD3 rs12946454	T	81.6	Ohasama	1583	0.356	99.4	1.76	0.72	**0.015**	0.99	0.48	**0.038**
		83.0	Yokohama	2274	0.517	99.3	0.23	0.56	0.687	0.12	0.37	0.752
		83.3	Shigaraki	2242	0.966	98.6	0.46	0.72	0.524	0.76	0.46	0.094
		85.3	Takashima	1712	0.707	99.0	-1.37	0.98	0.163	-1.09	0.56	0.052
		83.2	Suita	2528	0.234	99.7	0.53	0.73	0.464	0.08	0.42	0.845
		82.4	Matsuyama	805	0.799	99.6	0.34	1.28	0.790	0.86	0.82	0.290
		82.4	Nomura	2861	0.142	99.5	-0.35	0.75	0.635	-0.05	0.42	0.899
PLEKHA7 rs381815	T	15.1	Ohasama	1590	0.566	99.9	0.22	0.79	0.778	0.23	0.52	0.657
		19.7	Yokohama	2281	0.457	99.6	-0.77	0.52	0.139	0.04	0.35	0.900
		19.3	Shigaraki	2248	0.587	98.9	-0.38	0.68	0.574	-0.90	0.43	**0.034**
		19.0	Takashima	1719	0.434	99.4	-0.196	0.87	0.271	-0.22	0.50	0.660
		20.2	Suita	2527	0.421	99.6	0.76	0.69	0.272	0.42	0.40	0.289
		20.2	Matsuyama	808	0.496	100.0	0.99	1.19	0.408	0.53	0.76	0.489
		23.2	Nomura	2859	0.007	99.4	0.88	0.66	0.187	0.73	0.37	0.052
CSK-ULK3 rs6495122	A	79.4	Ohasama	1581	0.050	99.3	-0.39	0.69	0.569	-0.46	0.45	0.308
		78.4	Yokohama	2288	0.157	99.9	0.88	0.51	0.086	0.66	0.34	0.055
		83.5	Shigaraki	2237	0.146	98.4	0.96	0.72	0.183	0.96	0.45	**0.034**
		80.6	Takashima	1720	0.221	99.4	0.03	0.86	0.969	0.06	0.49	0.907
		81.6	Suita	2529	0.004	99.7	0.87	0.69	0.211	0.18	0.40	0.654
		81.5	Matsuyama	806	0.734	99.8	1.35	1.24	0.276	0.68	0.79	0.391
		82.6	Nomura	2855	0.115	99.3	1.16	0.75	0.120	0.64	0.42	0.129

Table S10 Continued

SNP	coded allele		cohort				SBP			DBP		
	allele	%	name	n	HWE	CR	coefficient	SE	p	coefficient	SE	p
JLK4 rs9815354	A	14.9	Ohasama	1569	0.749	98.6	-0.08	0.80	0.918	0.32	0.53	0.543
		10.5	Yokohama	2269	0.122	99.1	-1.01	0.67	0.134	-0.44	0.45	0.331
		12.7	Shigaraki	2252	0.099	99.1	-1.58	0.80	**0.047**	-0.10	0.50	0.846
		12.0	Takashima	1710	0.201	98.8	-0.57	1.08	0.600	0.15	0.62	0.802
		11.9	Suita	2521	0.456	99.4	-1.03	0.86	0.232	-0.08	0.49	0.867
		11.4	Matsuyama	804	0.389	99.5	-0.91	1.50	0.547	0.70	0.96	0.467
		9.1	Nomura	2853	0.632	99.2	0.79	1.00	0.427	1.21	0.56	**0.030**

Coefficients and standardized error for systolic and diastolic BP were calculated under additive model using multiple regression analysis adjusted for age, age2, sex, BMI. Adjustment for treatment with antihypertensive medication was achieved by adding fixed constants to measured values (+15mmHg for SBP and +10mmHg for DBP).

Table S11 Multiple linear regression analysis for BP trait and hypertension

Parameters	Coded allele	Systolic blood pressure			Diastolic blood pressure			Hypertension	
		Coefficient	Standardized coefficient	P	Coefficient	Standardized coefficient	P	Odds (95% C.I.)	p
Sex		2.38	0.05	<0.001	3.15	0.12	<0.001	1.33 (1.18-1.50)	<0.001
Age (years)		0.31	0.19	<0.001	0.96	1.03	<0.001	1.15 (1.12-1.19)	<0.001
Age^2		0.00	0.25	<0.001	-0.01	-0.74	<0.001	0.99 (0.99-0.99)	0.008
Body mass index (kg/m^2)		1.80	0.25	<0.001	1.12	0.27	<0.001	1.28 (1.26-1.30)	<0.001
Habitual drinking		0.79	0.02	0.035	0.93	0.04	<0.001	1.24 (1.11-1.40)	<0.001
ATP2B1 rs11105378	C	1.32	0.04	$4.4*10^{-8}$	0.71	0.04	$6.1*10^{-7}$	1.21 (1.12-1.30)	$4.0*10^{-7}$
FGF5 rs1458038	T	1.36	0.04	$1.5*10^{-8}$	0.77	0.04	$6.4*10^{-8}$	1.20 (1.11-1.29)	$1.4*10^{-6}$
CYP17A1 rs1004467	A	0.97	0.03	$8.9*10^{-5}$	0.35	0.02	0.017	1.14 (1.06-1.23)	$8.4*10^{-4}$
CSK rs1378942	C	0.71	0.02	0.014	0.36	0.02	0.036	1.09 (1.00-1.19)	0.046

Coefficients for systolic and diastolic BP were calculated using multiple linear regression analysis adjusted cohort variables. Adjustment for treatment with antihypertensive medication was achieved by adding fixed constants to measured values (+15mmHg for SBP and +10mmHg for DBP). Hypertensive subjects were defined as being treated with antihypertensive medication, or SBP greater or equal to 140 mmHg, or DBP greater or equal to 90 mmHg; normotensive subjects were defined as all of not treated with antihypertensive medication, and SBP less or equal to 120 mmHg, and DBP less or equal to 85 mmHg [8].

FIGURE S1

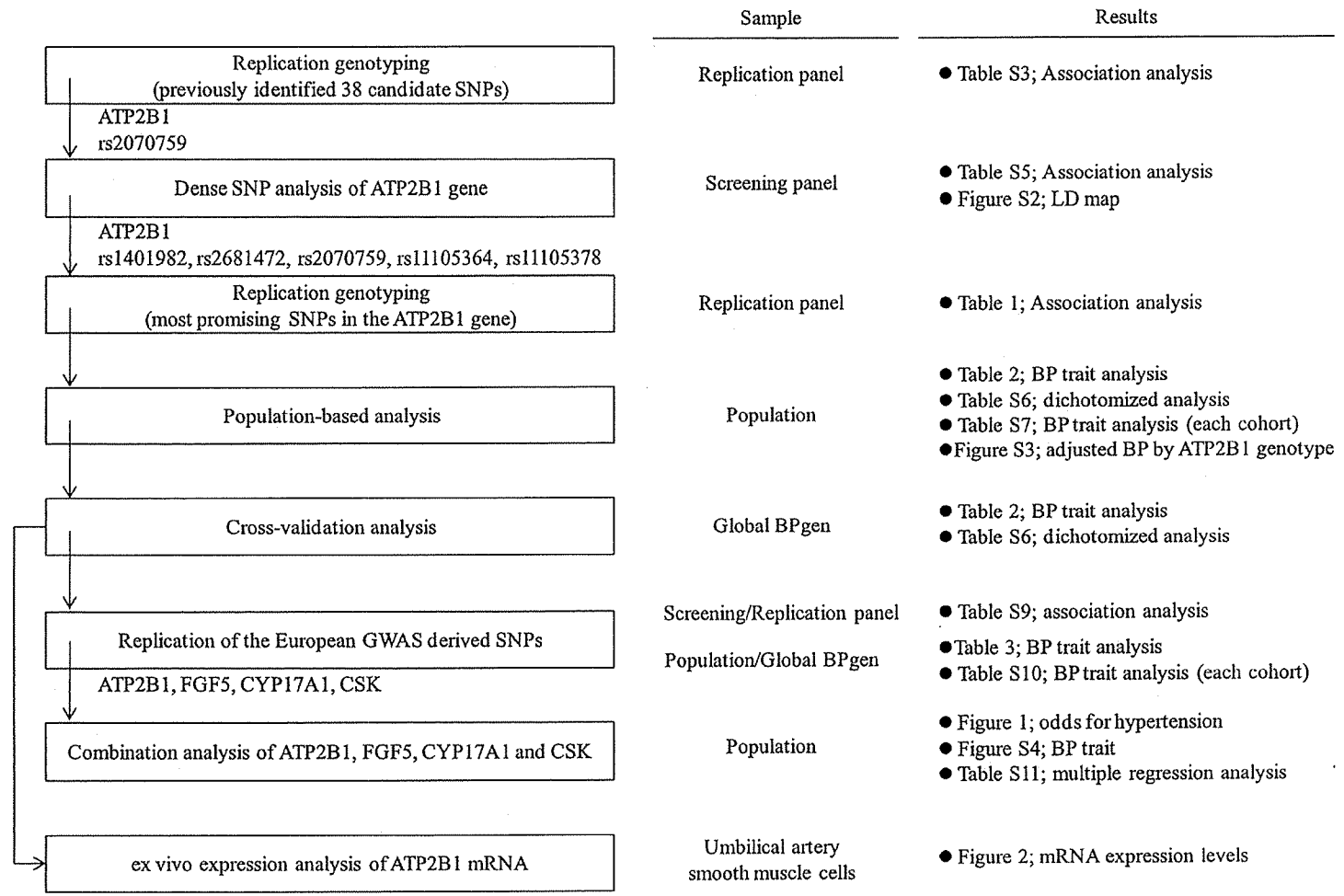

Figure S1 Study procedure and corresponding samples and results

FIGURE S2

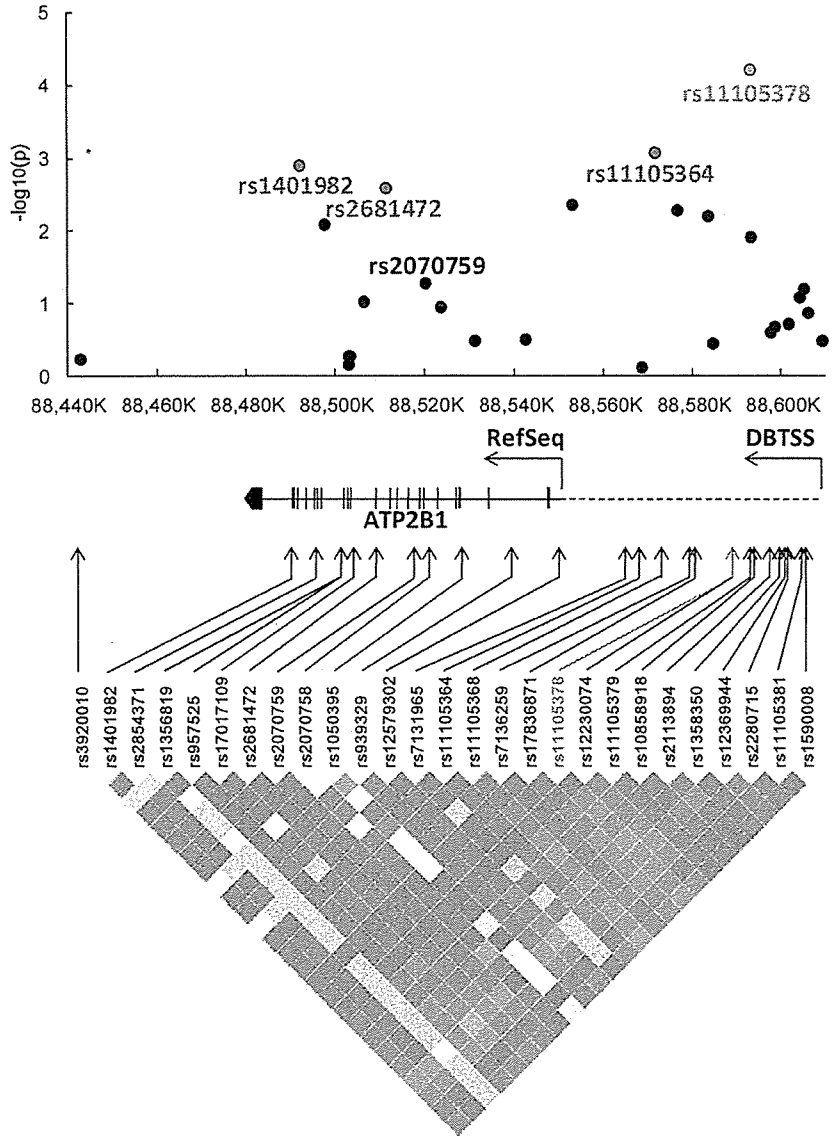

Figure S2 Dense SNP analysis of the ATP2B1 gene

The top graph shows p-values (-log10(P)) of association analyses using the screening panel (Table S4). The red circle (rs11105378) indicates the SNP showing the most significant association with hypertension. The lower panel shows a LD (D') map based on the genotype frequency of the control subjects

FIGURE S3

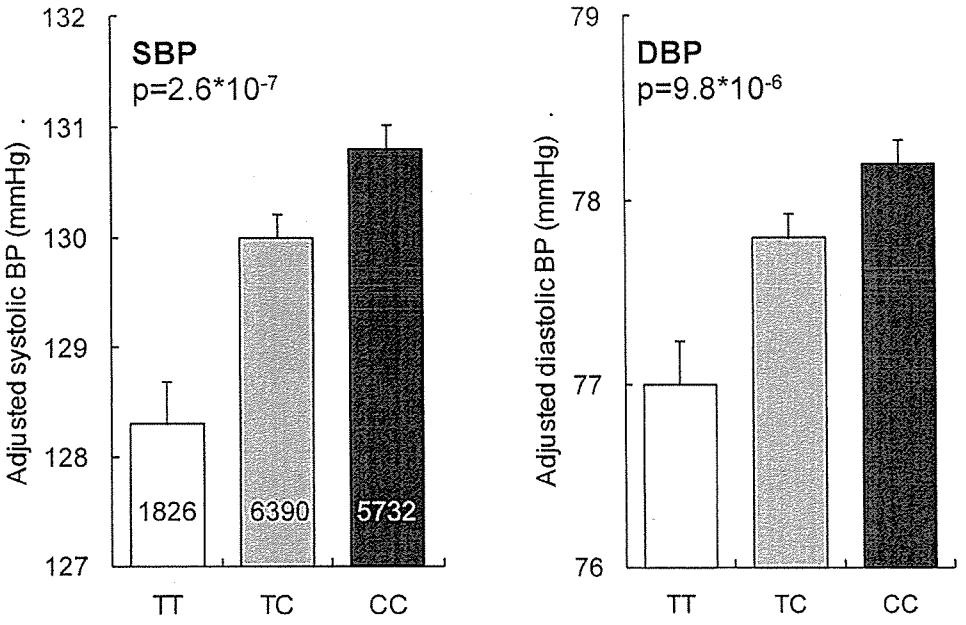

Figure S3 Adjusted systolic and diastolic BP among rs11105378 genotype

Values are mean±standard error adjusted for age, sex, body mass index, and cohort variables. Number of subjects in each genotype is represented in column.

FIGURE S4

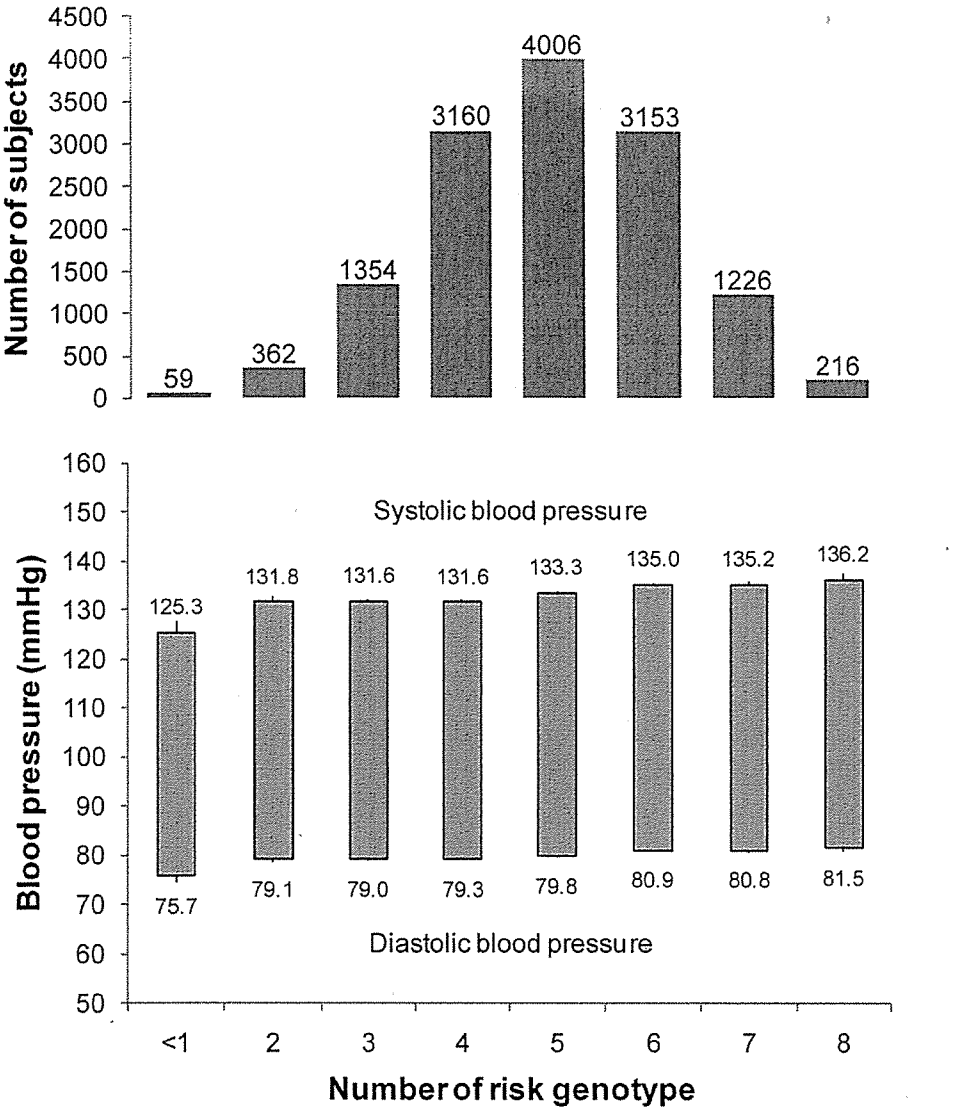

Figure S4 Adjusted blood pressure by the number of risk genotypes

Number of risk genotype was calculated by the following four SNPs; *ATP2B1* rs1105378, *FGF5* rs1458038, *CYP17A1*, rs1004467 and *CSK* rs1378942. Age, age2, sex, BMI and cohort variable adjusted systolic and diastolic BP is shown in the lower panel. Upper panel indicates the number of subjects in each group.

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