molecules were involved in the inhibition of atherosclerotic lesion area and lipid area induced by amlodipine. To clarify the importance of blockade of NADPH oxidase activity in the inhibitory action of amlodipine, future experiments using an inhibitor of NADPH oxidase might be very useful.

In summary, a CCB, amlodipine, not only inhibited the progression of atherosclerotic lesion formation, but also induced regression of atherosclerotic lesions at least in part due to inhibition of the inflammatory response and oxidative stress. The results of the present study may suggest a possible mechanism by which amlodipine inhibits the onset of cardiovascular events in patients with atherosclerosis.

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Original Article

Sex-Related Differences in the Relations of Insulin Resistance and Obesity to Left Ventricular Hypertrophy in Japanese Hypertensive Patients

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Echocardiographically determined left ventricular (LV) hypertrophy is a powerful, independent predictor of cardiovascular morbidity and mortality. Both insulin resistance and obesity have a well-known association with LV hypertrophy. However, whether or not there are sex-related differences in the relations of insulin resistance and obesity to LV hypertrophy has never been systematically explored in Japan. We enrolled 91 never-treated hypertensive patients (49 men and 42 women) to assess the possible relations of insulin resistance and obesity to LV geometry. Insulin resistance was estimated using the homeostasis model assessment (HOMA) formula. Echocardiographically determined LV mass and relative wall thickness were measured as markers of LV geometry. In addition, body mass index (BMI) was calculated as weight (kg) divided by height (m)^2 as a marker of obesity. Independent determinants of LV mass in male hypertensive patients were HOMA value ($p<0.0001$) and age ($p=0.034$). BMI did not bear a significant relation to LV mass. In comparison, in female hypertensive patients BMI was an independent determinant of LV mass ($p=0.011$). The HOMA value did not bear a significant relation to LV mass in the female hypertensive patients. In conclusion, these findings indicate the presence of sex-related differences in the relations of insulin resistance and obesity to LV hypertrophy in Japanese hypertensive patients. The effect of obesity on LV geometry was greater in female hypertensive patients than in male hypertensive patients. (*Hypertens Res* 2006; 29: 499–504)

Key Words: **essential hypertension, insulin resistance, obesity, left ventricular mass, gender**

Introduction

Echocardiographically determined left ventricular (LV) hypertrophy is known to be a powerful, independent risk factor of future cardiovascular morbidity and mortality in essential hypertension (*1–3*). Furthermore, there is increasing evidence of a link between LV hypertrophy and hypertensive target organ damage (*4–6*). The mechanisms through which LV hypertrophy increases cardiovascular risk are only partially understood, but might involve increased insulin resistance, which is increasingly recognized as an important predictor of cardiovascular morbidity and mortality (*7, 8*).

The Framingham Heart Study (*9*), a cross sectional study of 3,799 participants, found that LV mass and wall thickness increased with worsening glucose intolerance, an effect that was more striking in women compared with men. This relation was largely accounted for by obesity. The combination of

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Table 1. Patients Characteristics

	Male hypertensive patients (n=49)	Female hypertensive patients (n=42)
Age (years)	59±12	63±9
Pulse rate (beats/min)	68±12	70±10
Blood pressure (mmHg)		
Systole	161±14	159±14
Diastole	90±12	87±10
Pulse pressure (mmHg)	71±13	72±15
Body mass index (kg/m^2)	24.5±2.9	23.9±3.6

Values are mean±SD.

Table 2. Sex-Related Differences in Biochemical Characteristics in Hypertensive Patients

	Male hypertensive patients (n=49)	Female hypertensive patients (n=42)
Fasting plasma glucose (mmol/l)	5.44±0.78	5.33±0.50
Fasting immunoreactive insulin (pmol/l)	53.81±24.40	41.61±15.79*
HOMA value	1.87±0.99	1.38±0.62*
Total cholesterol (mmol/l)	4.99±0.83	5.59±1.01*
HDL-cholesterol (mmol/l)	1.14±0.34	1.40±0.41*
Triglycerides (mmol/l)	1.58±0.63	1.38±0.45

Values are mean±SD. *p<0.01 *vs.* male hypertensive patients. HOMA, homeostasis model assessment; HDL, high-density lipoprotein.

obesity and hypertension is more consistently associated with LV hypertrophy than either stimulus alone (*10*). Furthermore, we have previously reported that there is a sex-related difference in the relation of serum uric acid level and LV mass in hypertensive patients (*11*). Although an association of increased LV mass with adverse outcomes has been consistently reported in men and women, whether or not the relative impacts of insulin resistance and obesity on the prevalence of LV hypertrophy are similar in the two sexes has never been systematically explored in Japan.

Accordingly, we examined the sex-related differences in the relations of insulin resistance and obesity to LV hypertrophy identified by echocardiographically determined LV mass in nondiabetic and never-treated patients with essential hypertension.

Methods

Study Population

The study population included 91 nondiabetic patients with essential hypertension (49 men and 42 women; mean age: 61±10 years old). They had normal findings on a chemical screening battery and were nondiabetic by the criteria of the American Diabetes Association (*12*). All study patients participated in this study after giving informed consent. The study was carried out in accordance with the Declaration of Helsinki (1989) of the World Medical Association. To exclude the presence of secondary forms of hypertension, all patients underwent a complete medical history, physical examination, and appropriate laboratory evaluation (*4*).

Physical Examinations

Physical examinations in hypertensive patients were supervised by the nursing staff. Weight and height were measured while the subjects were fasting overnight and wearing only underwear. Body mass index (BMI) was calculated as weight (kg) divided by height (m)^2. Blood pressure (BP) was measured in triplicate by a single physician who was expert in the evaluation of hypertension, with an appropriate arm cuff and a mercury sphygmomanometer after 5 min of rest in the sitting position. The arithmetic mean of the last two measurements was calculated. Korotkoff phase V was taken for diastolic blood pressure. Hypertension was defined as systolic BP (SBP) equal to or greater than 140 mmHg and/or diastolic BP (DBP) equal to or greater than 90 mmHg (*13*).

Biochemical Measurements

In the morning, after an overnight fast, venous blood was sampled for the measurement of plasma concentrations of glucose and insulin, and serum concentrations of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and triglycerides (TG). Plasma glucose was immediately determined by the glucose oxidase method. Plasma insulin was determined in duplicate by a highly specific and sensitive immunoradiometric assay (Abbott Japan; intraassay coefficient of variation (CV): 1.6%; interassay CV: 2.2%). Serum concentrations of TC, HDL-C and TG were assessed by standard enzymatic methods.

Insulin resistance was assessed from fasting immuno-reactive insulin (FIRI) and fasting plasma glucose (FPG) using the previously validated homeostasis model assessment (HOMA) (*14*) according to the following formula: HOMA value = FIRI (pmol/l) × FPG (mmol/l)/161.

Echocardiographic Measurements

Two-dimensionally guided M-mode echocardiography was performed by standard methods as previously outlined (*4*) using an SSD-6500 echocardiograph with a 3.5 MHz transducer (Aloka Inc., Tokyo, Japan). Echocardiographic examinations were performed and interpreted by the same

Table 3. Sex-Related Differences in Echocardiographic Characteristics in Hypertensive Patients

	Male hypertensive patients (n=49)	Female hypertensive patients (n=42)
LVM (g)	203±47	154±35#
LVM index (g/m²)	120±26	102±21**
Relative wall thickness	0.41±0.10	0.37±0.07*
Percent FS (%)	36.5±7.0	38.1±6.5
SV/PP ratio	1.11±0.33	1.05±0.29

Values are mean±SD. *p<0.01, **p<0.001, and #p<0.0001 *vs.* male hypertensive patients. LVM, left ventricular mass; FS, fractional shortening; SV, stroke volume; PP, pulse pressure.

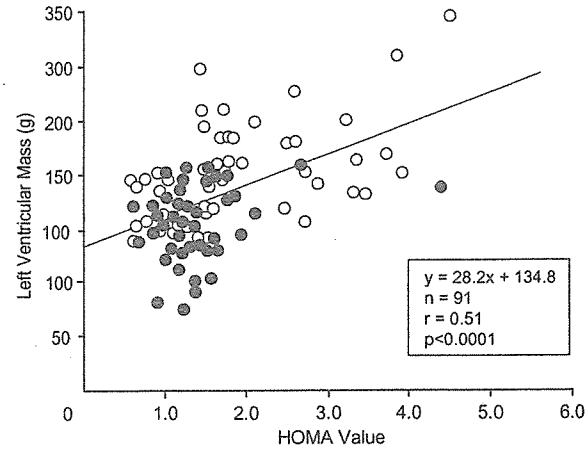

Fig. 1. *Relationship between the HOMA value and echocardiographically determined left ventricular mass in male hypertensive patients (open circles) and female hypertensive patients (closed circles). A statistically significant positive relation was found between the HOMA value and left ventricular mass in all hypertensive patients.*

cardiologist, who was unaware of the patient's data. LV internal dimension (LVID) and interventricular septal thickness (IVST) and posterior wall thickness (PWT) were measured at end-diastole and end-systole, according to the American Society of Echocardiography guidelines (*15*). LV mass was calculated according to a necropsy-validated formula (*16*). LV mass was also indexed by body surface area (BSA). Relative wall thickness (RWT) was measured as follows: RWT = 2 × (PWTd/LVIDd), where d is end-diastole. Percent fractional shortening (FS) was calculated as (LVIDd – LVIDs)/LVIDd × 100 and was used as an indicator of LV systolic function, where d and s are end-diastole and end-systole, respectively. End-diastolic and end-systolic LV volumes were calculated by the Teichholz method (*17*) using linear measurements at diastole and systole; this method has been validated by invasive and Doppler reference standards. Stroke volume (SV) was calculated as (end-diastolic LV volume – end-systolic LV volume). The ratio of SV to pulse pressure (PP) was used as an indirect measure of aortic compliance (*18*).

Statistical Analysis

All values are expressed as the mean±SD. Two-tailed unpaired Student's t-test was used to compare study response variables between categories. Correlation coefficients were calculated according to Pearson's method. A multiple regression analysis was also performed to select appropriate independent variables producing the highest partial correlation with LV mass in hypertensive patients. Probability values <0.05 were considered statistically significant in all analyses.

Results

Sex-Related Differences in Demographic and Clinical Characteristics

There were no significant differences in age, pulse rate, SBP, DBP, PP, and BMI between male and female hypertensive patients (Table 1).

Sex-Related Differences in Biochemical Characteristics

Although there was no significant difference in FPG between male and female hypertensive patients, FIRI and HOMA values in male hypertensive patients were significantly higher than those in female hypertensive patients. Both TC and HDL-C levels in female hypertensive patients were significantly higher than those in male hypertensive patients. There was no significant difference in TG level between the two hypertensive groups (Table 2).

Sex-Related Differences in Echocardiographic Characteristics

LV mass, LV mass index and RWT in male hypertensive patients were significantly larger than those in female hypertensive patients. There were no significant differences in percent FS and SV/PP ratio between male and female hypertensive patients (Table 3).

Subgroups Analysis

On the basis of the relationship between RWT and LV mass index, the 49 male and 42 female hypertensive patients were then divided into concentric, eccentric, and other hypertrophy groups. The partition values of 0.44 for RWT and 108 g/m² (male) or 104 g/m² (female) for LV mass index, which were

Table 4. Simple Correlation of Left Ventricular Mass with Demographic, Biochemical, and Ehchocardiographic Variables in Male and Female Hypertensive Patients

	Left ventricular mass			
	Male hypertensive patients (n=49)		Female hypertensive patients (n=42)	
	r values	p values	r values	p values
Age	0.224	0.1214	0.104	0.5122
Body mass index	0.138	0.3436	0.370	0.0157
Systolic blood pressure	0.218	0.1332	0.025	0.8755
Pulse pressure	0.056	0.7044	0.020	0.9017
Fasting plasma glucose	0.064	0.6636	0.083	0.6005
Immunoreactive insulin	0.563	<0.0001	0.319	0.0395
HOMA value	0.502	0.0002	0.278	0.0744
Precent fractional shortening	0.256	0.0755	0.177	0.2631
Stroke volume/pulse pressure ratio	0.070	0.6340	0.294	0.0586

HOMA, homeostasis model assessment.

Table 5. Multiple Regression Analysis of Factors Relevant to Left Ventricular Mass in Male and Female Hypertensive Patients

	Left ventricular mass					
	Male hypertensive patients (n=49)			Female hypertensive patients (n=42)		
	β	r values	p values	β	r values	p values
Age	0.265	2.193	0.034	0.149	1.020	0.314
Body mass index	0.058	0.482	0.632	0.392	2.680	0.011
Systolic blood pressure	0.193	1.607	0.115	0.018	0.125	0.901
HOMA value	0.526	4.336	<0.0001	0.269	1.852	0.072
	Multiple r^2=0.373, p=0.0003			Multiple r^2=0.236, p=0.037		

HOMA, homeostasis model assessment.

the mean + 2SD value of normotensive control subjects, were used (*4*). In male hypertensive patients, there were 17 (35%) patients with concentric hypertrophy and 15 (31%) with eccentric hypertrophy; in female hypertensive patients, there were 3 (7%) patients with concentric hypertrophy and 16 (38%) with eccentric hypertrophy. The prevalence of concentric hypertrophy in male hypertensive patients was significantly higher than that in female hypertensive patients.

Relations of Insulin Resistance, Demographic Factors and Percent FS to LV Mass

Figure 1 shows the relationship between the HOMA value and echocardiographically determined LV mass in hypertensive patients. As shown in Table 4, LV mass was significantly related to HOMA value and FIRI in male hypertensive patients. However, LV mass was related to BMI and FIRI in female hypertensive patients. In both sexes, LV mass was not related to age, SBP, PP, FPG, percent FS, or SV/PP ratio.

Table 5 shows the results of multiple regression analysis. Independent determinants of LV mass in male hypertensive patients were age and HOMA value. BMI did not bear a significant relation to LV mass. In contrast, in female hypertensive patients BMI was an independent determinant of LV mass. The HOMA value did not bear a significant relation to LV mass in the female hypertensive patients.

Discussion

In this cross-sectional study, LV mass correlated positively with the HOMA value in male hypertensive patients, but not in female hypertensive patients. In comparison, LV mass correlated positively with BMI in female hypertensive patients, but not in male hypertensive patients. These findings indicate the presence of sex-related differences in the relations of insulin resistance and obesity to LV hypertrophy in Japanese hypertensive patients.

It is widely acknowledged that peripheral hyperinsulinemia in patients with hypertension is a marker of insulin resistance (*19*, *20*). Bonora *et al.* (*21*) reported that diminished insulin sensitivity with regard to glucose utilization causes a substantial increase of insulin production in an attempt to maintain normal glucose utilization, making it possible that cardiovascular trophic effects and other actions of insulin could be exaggerated. They therefore calculated the HOMA value in order to obtain a better quantitative estimate of insulin resistance (*21*). In the present study, we showed an independent association between echocardiographically determined LV

mass and the HOMA value in male hypertensive patients, but not in female hypertensive patients. A potential limitation of the present study is that the insulin levels were assessed in the fasting state but not in response to glucose loading. Several studies have found a positive association between postload insulin levels or area under the postload insulin curve and LV structural variables (*22–24*).

In a recent investigation, the HOMA value was related to LV mass in women alone, but this relation was largely accounted for by obesity (*9*). In the present study, on the other hand, the HOMA value was related to LV mass in male hypertensive patients, but not in female hypertensive patients. Furthermore, this relation was not accounted for by BMI. If there is a sex-related difference in the impact of insulin resistance on LV mass, the underlying mechanism is unclear. One possibility is that insulin may have variable effects on LV geometry according to gender and race.

Verdecchia *et al.* (*24*) have reported that insulin and insulin growth factor-1 (IGF-1) were powerful independent determinants of LV mass in nondiabetic patients with hypertension. The direct effect of insulin on cardiac myocyte growth could be mediated at least in part, by IGF-1 receptors (*25*). Unfortunately, we were not able to measure IGF-1 binding protein in the present study. However, because the fasting insulin level was positively correlated to LV mass, our data suggest that insulin is a powerful determinant of cardiac myocyte growth in untreated patients with essential hypertension and normal glucose tolerance. In addition, hypertensive patients with glucose intolerance have more severe LV hypertrophy and LV diastolic dysfunction than those with normal glucose tolerance (*26*, *27*).

Obesity had a major impact on the development of LV hypertrophy in our female hypertensive patients. The increase in LV mass was statistically independent of age, blood pressure and insulin resistance. As expected from previous reports (*10*, *28*, *29*), the most prevalent LV geometric abnormality in obese patients with hypertension was eccentric LV hypertrophy. In the present study, although the most prevalent LV geometric abnormality in male hypertensive patients was concentric LV hypertrophy, the most prevalent LV geometric abnormality in female hypertensive patients was eccentric LV hypertrophy, confirming that the effect of obesity on cardiac anatomy is greater in women than in men (*30*).

Our multivariate analyses showed that the likelihood of LV hypertrophy identified by LV mass increases with age in male hypertensive patients. On the other hand, de Simone *et al.* have previously reported that increase in LV mass with age in women was associated with hemodynamic and hormonal changes that were not evident in men, suggesting a volume expansion occurring after menopause (*31*). Furthermore, the LV wall thickness and LV mass have been shown to significantly increase with advancing age in healthy normotensive subjects (*32*, *33*). Therefore, a possible explanation for the absence of an association between LV mass and age in our female hypertensive patients would be the small sample size. Another potential limitation of this study is its cross-sectional nature; in the future, it would be useful to perform a cardiovascular evaluation of individuals with previous serial data on LV geometry.

In conclusion, there is increasing evidence of a link between insulin and cardiovascular risk (*7*), although the independent role of insulin is still undetermined. The present study indicated the presence of sex-related differences in the relations of insulin resistance and obesity to LV hypertrophy in Japanese hypertensive patients. The effect of obesity on LV geometry was greater in female hypertensive patients than in male hypertensive patients.

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High-Resolution Mapping for Essential Hypertension Using Microsatellite Markers

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Abstract—During the past decade, considerable efforts and resources have been devoted to elucidating the multiple genetic and environmental determinants responsible for hypertension and its associated cardiovascular diseases. The success of positional cloning, fine mapping, and linkage analysis based on whole-genome screening, however, has been limited in identifying multiple genetic determinants affecting diseases, suggesting that new research strategies for genome-wide typing may be helpful. Disease association (case–control) studies using microsatellite markers, distributed every 150 kb across the human genome, may have some advantages over linkage, candidate, and single nucleotide polymorphism typing methods in terms of statistical power and linkage disequilibrium for finding genomic regions harboring candidate disease genes, although it is not proven. We have carried out genome-wide mapping using 18 977 microsatellite markers in a Japanese population composed of 385 hypertensive patients and 385 normotensive control subjects. Pooled sample analysis was conducted in a 3-stage genomic screen of 3 independent case–control populations, and 54 markers were extracted from the original 18 977 microsatellite markers. As a final step, each single positive marker was confirmed by individual typing, and only 19 markers passed this test. We identified 19 allelic loci that were significantly different between the cases of essential hypertension and the controls. (***Hypertension.* 2007;49:446-452.)**

Key Words: essential hypertension ■ genome-wide ■ association study ■ Japanese ■ new candidate regions

Hypertension is a leading risk factor for cerebrovascular disease, coronary heart disease, and renal failure.[1] It is the major cause of morbidity and mortality and also the third highest risk factor for lifetime burden worldwide.[2,3] Kearney et al[4] reported that there were 972 million hypertension patients in the world, accounting for 26.4% of the adult population in 2000, and predicted that this figure will increase to 1.56 billion patients (29.2%) by 2025. The present pandemic of cardiovascular diseases has been attributed largely to the high prevalence of hypertension, suggesting that more emphasis should be placed on the prevention, detection, and treatment of hypertension.

Elucidation of the genetic etiology of hypertension has been increasingly emphasized as important for a better understanding of the pathogenesis of this disease and for ultimately improving the prevention strategies, diagnostic tools, and therapy in the new millennium.[5] Hypertension is one of the risk factors for coronary heart disease, which is a common complex human genetic disease, and its genetic variance accounts for 30% to 70% of the trait variance.[6,7] The sibling recurrent risk ratio of hypertension is reportedly to be 2 to 3.[8] Each of the hypertension-causing gene recurrent risk ratios is less than the aggregate sibling recurrent risk ratio. There are now many reports describing the results of genome-wide screens for genes controlling blood pressure (BP). The National Heart, Lung, and Blood Institute Genelink project website (https://genelink.nhlbi.nih.gov) lists the National Heart, Lung, and Blood Institute–supported genome scans for BP. The majority of these reports have described numerous chromosomal regions with suggestive evidence of linkage.[9] However, the application of linkage analysis to hypertension, with the exception of obvious Mendelian inheritance, has achieved only limited suc-

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cess thus far.[10,11] Since 2000, ≥6 large genome scans have been reported,[12] namely, an admixture mapping study,[13] a Medical Research Council Program-funded British Genetics of Hypertension Study,[14] the US National Institutes of Health-funded Family Blood Pressure Program studies,[15–18] the Victorian Family Heart Study,[19] the San Antonio Heart Study,[20] and the Quebec Family Study.[21] Except for the admixture mapping study, all of these studies were based on linkage analysis.

In many cases, complex diseases are complicated by genetic heterogeneity and small effects of each gene. In 1996, Risch and Merikangas reported[22] that numerous genetic effects in complex diseases were too weak to be identified by linkage analysis and could be better detected by genomic association studies. Thus, the new challenges to identify disease-predisposing variants in human genome research have resulted in approaches, such as the Hapmap project,[23] high-density single nucleotide polymorphism (SNP) analysis,[24] and microsatellite (MS) association analysis.[25] Disease association studies using MS markers distributed across the human genome every 100 to 150 kb have distinct advantages over linkage analysis, the candidate approach, and SNP typing in terms of linkage disequilibrium (LD).[25] The MS markers are highly polymorphic, showing a high degree of heterozygosity (on average, ≈70%) and LD lengths in the 100- to 200-kb range.[26–31] As compared with MS markers, SNPs have a low degree of genetic polymorphism (biallelic) and have a shorter, by ≈30 kb, LD range, probably because of their older age. Varilo et al[32] reported that highly polymorphic MS markers can provide much greater power for detection of intermarker LD than can either single SNPs or SNP haplotypes on chromosomes 1q and 5q. Therefore, it is possible to carry out substantial whole genome association analysis using a smaller number of MS markers than SNPs (eg, tens of thousands of MS markers versus hundreds of thousands or millions of SNPs). Recently, the usefulness of the haplotype approach and haplotype-tagging SNPs from the HapMap project has been questioned.[33]

Methods

Subjects for MS Typing

A total of 425 (stage 1: 95; stage 2: 131; stage 3: 199) patients with essential hypertension and 467 (stage 1: 103; stage 2: 132; stage 3: 232) normotensive healthy individuals participated in this study. The number of subjects for pooled DNA typing was 95 versus 95 for stage 1, 120 versus 120 for stage 2, and 170 versus 170 for stage 3. After pooled DNA typing, individual typing for the same samples was performed. The difference in number in each stage was derived from the time of sample collection. It was aimed to collect 100 volunteers for each stage each in case and control subjects, but many more subjects were collected beyond our expectations. So, we made the most of all of the subjects to increase the statistical power. The subjects were of Japanese origin from Hokkaido, Tokyo, Kanagawa, Shiga, Osaka, Kyoto, and Ehime. The subjects for the stage 1 and stage 2 screens were recruited from the Millennium Genome Project, and the subjects for the stage 3 screen were recruited from Yokohama City University School of Medicine. The diagnosis of essential hypertension was made according to the guidelines of the Japanese Society of Hypertension (declared in 2000), which include a sitting systolic BP of >140 mm Hg and/or diastolic BP of >90 mm Hg on ≥2 occasions after the first medical examination. Furthermore, subjects in this study were selected as follows, as shown in Table 1. Our criteria were classification as moderate or severe hypertension.

TABLE 1. Characteristics of Subjects

Character	Hypertensive Patients	Normotensive Control Subjects
Gender	Male	Male
Age of onset	30 to 59	≥50
Family history	Within parents and siblings	None
Body mass index, kg/m^2	≤25	≤25
BP, mm Hg	Systolic BP≥160 and/or diastolic BP≥100	Systolic BP≤120 and diastolic BP≤80 and no antihypertensive treatment

We obtained informed consent from all of the patients and healthy individuals whose DNA samples were used in the analyses. Our experimental procedures were approved by the relevant ethical committee in each participating university and center. All of the personal identities associated with medical information and blood samples were carefully eliminated and replaced with anonymous identities in each recruiting institution.

Pooled DNA and Genotyping

Ninety-five subjects in stage 1, 120 in stage 2, and 170 in stage 3 were selected from each group (case and control subjects) based on the DNA quality and quantity for DNA pooling analysis. The DNA pooling method was adopted to bring down the cost and the technical burden linked to genotyping thousands of MSs without losing any significant amount of data.

The DNA pooling method for MS typing was carried out by making slight modifications[34] of the protocol of Collins et al.[35] The key factor in this methodology is the absolute equality of individual DNA quantities, so we used a highly accurate quantitative procedure to construct a pooled DNA template for PCR amplification.[35] This pool was composed of strictly measured DNA concentrations, extracted from 95 stage 1, 120 stage 2, and 170 stage 3 Japanese individuals. We checked each DNA concentration ≥3 times and equalized each DNA concentration by dilution. Multiple peak patterns in the pooled DNA showed the distribution of allele frequencies in the subjects.[35] The DNA pooling method enabled us to obtain the allele frequencies of MSs in pooled Japanese individuals by measuring the heights of multiple peaks and to apply this approach to an association study. The quality of the pooled DNA was confirmed by comparing the allelic distributions between individual and pooled typing results using 23 MS markers, unless there was the absence of any significant difference ($P \leq 0.05$) in allele frequencies between pooled DNA typing and individual. This comparison of allele frequencies for the same allele was performed by Fisher's exact test.

DNA was extracted using a QIAamp DNA blood kit (Qiagen) under standardized conditions to prevent variation in DNA quality. This was followed by 0.8% agarose gel electrophoresis to check for DNA degradation and RNA contamination. After measurement of the optical density to check for protein contamination, the DNA concentration was determined through 3 successive measurements using the PicoGreen fluorescence assay (Molecular Probes). Standardized pipetting and aliquoting of the DNA samples were robotically performed using a Biomek 2000 and Multimek 96 (Beckman). The pooled DNA template for typing with 2×18 977 MS markers (first set: case subjects; second set: control subjects) was prepared immediately after DNA quantification. After the initial tests, the 18 977 PCR reaction mixtures containing all of the components except primers were prepared and then aliquoted into 96-well reaction plates and stored until use. The MS pooled typing and individual genotyping procedures after the PCR reaction were carried out according to standard protocols using ABI3700 and 3730 DNA analyzers (Applied Biosystems). The standardized preparations allowed the reproducibility and accuracy to be maintained for the pooled DNA typing throughout the experiment. Various kinds of

information, such as the peak positions and heights, were manually extracted by the PickPeak and MultiPeaks programs, developed by Applied Biosystems Japan, from the multipeak pattern in the chromatogram ABI fsa files.

In the first stage, 95 case and 95 control subjects were subjected to association analysis using all of the 18 977 markers. Among them, markers showing statistical significance of $P<0.05$ were subjected to the second stage with another 120 case subjects and 120 control subjects. The markers showing statistical significance of $P<0.05$ in the second screening were subjected to a third stage with another 170 case subjects and 170 control subjects. All of the positive markers that remained statistically significant ($P<0.05$) in the stage 3 screening were confirmed by individual genotyping using the same set of 385 case subjects and 385 control subjects as the final step.

Marker Information

MS sequences were computationally detected from all of the chromosomes except for the Y chromosome (in 4 versions of the human genome draft sequence: Golden Path Jun 2004 to the National Center for Biotechnology Information build 35). At present, our laboratory has built 27 037 markers as a full set.[25]

In this study, we used 18 977 markers with an average spacing of 145.9 kb (Tables I and II, available online at http://hyper.ahajournals.org.) from 19 654 markers in the first built set. The other 678 markers were excluded because of problems with the PCR reaction and marker quality.

The MS markers were investigated for repeat polymorphisms in 200 healthy Japanese by using the DNA pooling method. Our criteria for selection of MS markers for the hypertension association study were dinucleotide repeats with >10 repeats; tri-, tetra-, and pentanucleotide repeats with >5 repeats; and polymorphic MS markers with heterozygosity of >30% but not those with heterozygosity of >85% to eliminate any unstable and highly mutated MS markers. We chose PCR primers that contained no SNPs in the sequences to prevent differential amplification. Seven PCR primer pairs of 54 MS markers for individual typing were redesigned to improve the efficiency of PCR (Table III). Detailed information on the 27 039 MS markers is available on the Japan Biological Information Research Center homepage (http://www.jbirc.aist.go.jp/gdbs/).

Statistical Analysis

The measurements of the heights of multiple peaks in the pooled DNA were applied to association analysis. To calculate P values, we used 2 types of Fisher's exact test for 2×2 contingency tables for each individual allele and 2×m contingency tables for each locus, where m refers to the number of marker alleles observed in a population. The Markov chain/Monte Carlo simulation method was used to execute Fisher's exact test for the 2×m contingency table. The simple "allelic" but not "genotype" association was presented for the 2×2 contingency tables for MSs. These analyses were executed using the software package, AStat. The method of Pritchard and Rosenberg[36] was used for the detection of stratification in case and control populations using 23 MS markers. The Hardy–Weinberg test for allele frequency distributions at the MS loci was performed by P test for differentiation, as determined by GenePop 3.4. Other basic analyses were carried out using Microsoft Excel.

The authors had full access to the data and take responsibility for its integrity.

Results

Three-Stage Screening: Pooled DNA Typing

Before the 3-stage screenings, we verified spurious associations through the method of Pritchard and Rosenberg[36] using 23 randomly selected MS markers from each of chromosomes 1 to 22 and X, with an absence of any significant stratification in either the case or control populations (data not shown). This test is important to prevent spurious associations by population stratifications, especially for late-onset diseases, such as essential hypertension.

We initially identified 54 markers as potential hypertension susceptibility loci by 3-stage screening of 3 independent case–control populations (stage 1: 95; stage 2: 120; stage 3: 170 patients with essential hypertension and normotensive healthy individuals; Table 2). Three-stage screenings were intended to sequentially replicate the results in the 3 independent sample populations and eliminate pseudopositive markers resulting from type I errors.[37,38]

The number of markers decreased from 18 977 to 1160 markers in the first screening, then to 284 markers in the second screening, and finally to 54 markers in the third screening. The significance ($P<0.05$) of the association of positive markers was assessed by the Fisher's exact test, using either 2×2 or 2×m (m=number of alleles) contingency tables. Both 2×2 and 2×m analysis were performed together at each marker. If either of the 2 had $P<0.05$, the marker was judged as a positive marker. Finally, 54 markers significant in all of the screening sets were significant in the 2×2 test, and some of the markers were significant in the 2×m test. The concordance between the 2×2 and 2×m tests was relatively low (stage 1: 60%; stage 2: 64%; stage 3: 81%). All of the positive markers were checked by the Hardy–Weinberg test for allele frequency distributions at the MS loci, and then significant markers ($P\leq0.05$) in the Hardy–Weinberg test were excluded. The positive rates in the second and third screenings were higher than that in the first. This might be partially because of experimental artifacts of the pooled DNA method as reported by Sham et al[39] and Shaw et al.[40]

Individual Typing

The results of pooled typing were presumptive, so we genotyped a total of 770 individuals (385 case subjects versus 385 control subjects) and reanalyzed the 54 markers in the 3-stage screening procedure. These individuals are the same individuals as used in pooled typing and were not from a new cohort. Ultimately, we reduced the number of positive markers from 54 to 19 loci by using individual genotyping in the genome-wide association study for hypertension (Table 2). All 19 of the markers were significant ($P<0.05$) by 2×2 analysis, but only 3 markers were also significant ($P<0.05$) by 2×m analysis. In addition, the odds ratios ranged from 0.13 to 1.8 (Table 2).

The 19 genomic loci were observed on chromosome 2, 3, 4, 6, 10, 13, 17, 18, 19, and 20 (Table 3). The observed and expected frequencies of each genotype for the 19 markers in the case and control subjects were in Hardy–Weinberg equilibrium (data not shown). In considering the LD range, the susceptibility genes for hypertension were estimated to reside in a 100- to 150-kb region from each marker. We have also provided a list of the genes that are known to be positioned closest to the centromeric and telomeric side of each marker (Table 3) to highlight the locations of the 19 positive markers. The chromosomal location of the 19 markers in our study (Table 3) was compared with those identified in previous studies (Table 4). Essentially, 3 chromosomal locations were found to overlap in comparison with other studies: chromosome locations 2p11.1-q12.3, 2p25.1, and 6q27.

TABLE 2. Summary of the Phased Genome Screen by the DNA Method and Individual Typing

	First Screening			Second Screening		Third Screening		Individual Typing	
Chromosome	No. of MS	No. of Positives	Positive Rate, %	No. of Positives	Positive Rate, %	No. of Positives	Positive Rate, %	No. of Positives	Positive Rate, %
1	1516	146	10	31	21	7	23	0	0
2	1847	112	6	29	26	5	17	3	60
3	1422	66	5	15	23	3	20	3	100
4	1157	69	6	13	19	7	54	2	29
5	1173	71	6	14	20	0	0	0	0
6	1204	89	7	22	25	7	32	1	14
7	1266	66	5	18	27	2	11	0	0
8	830	58	7	12	21	0	0	0	0
9	838	32	4	6	19	3	50	0	0
10	907	45	5	11	24	2	18	1	50
11	916	40	4	10	25	0	0	0	0
12	684	25	4	6	24	0	0	0	0
13	609	32	5	11	34	2	18	2	100
14	571	29	5	6	21	1	17	0	0
15	458	34	7	9	26	2	22	0	0
16	508	25	5	9	36	0	0	0	0
17	495	42	8	17	40	5	29	4	80
18	539	27	5	8	30	2	25	1	50
19	358	33	9	8	24	3	38	1	33
20	430	23	5	7	30	3	43	1	33
21	260	15	6	2	13	0	0	0	0
22	228	16	7	5	31	0	0	0	0
X	756	64	8	15	23	0	0	0	0
Y	5	1	20	0	0	0	0	0	0
Total	18,977	1160	6	284	24	54	19	19	35

P value<0.05 was set at statistical significance by Fisher's test for the 2×2 or 2×*m* contingency table. The positive rates represented the rate of the markers, which were positive in the 2×2 or the 2×*m* analysis, to analyzed 18 977 (first), 1160 (second), and 284 markers (third).

Most of the 35 markers that were eliminated by individual typing after the 3-stage screening procedure may have been experimental artifacts or pseudopositive markers because of the DNA pooling method, PCR assay conditions, faulty peak heights during electrophoresis, PCR ghost peaks because of dissociation of labeled fluorescent reagents from a primer oligonucleotide, complications resulting from stutter, and additional nucleotide bands inherent to a particular MS.

Discussion

High-Density MS Markers

We conducted a 3-stage genome-wide scan of 3 independent case–control populations by an association test using 18 977 MS markers to identify susceptible genes for essential hypertension. In this study, we used 18 977 markers with an average spacing of 145.9 kb as the first built set (Tables I and II). Based on recent knowledge, the average length of LD between the disease-susceptible SNPs and the nearby MS alleles is ≥100 kb.[26–31] In other words, if the disease-susceptible SNPs are harbored between 2 neighboring MS markers at an interval ≤200 kb, LD between the disease-susceptible SNPs and either of the nearby MS will be proved. The use of average spacing of genetic markers across 100 to 200 kb of the entire genome is the best practical solution in genome-wide association analysis before availability of a genome-wide LD map, because the LD pattern varies between different regions of the human genome depending on several factors, such as allele frequency, mutation, and recombination.[33] Therefore, our first step for genome-wide analysis was to collect enough MS markers (>18 000 MS, 1 MS at every 150 kb) to cover the euchromatic area (≈90%) of the human genome (3 giga base; 3×10^9 kb ×0.9/150 kb=18 000). The remaining part of the genome was mostly heterochromatin restricted mainly to centromeres and telomeres, rich in repetitive sequences and believed to lack expressed genes. This 150-kb spacing of MS markers would enable us to assure an average 75-kb LD interval, which was presumed to detect the presence of disease-susceptible loci flanked by 2 neighboring MS markers across the whole genome.

Although 54 MS markers were found significant in all 3 stages of the pooling experiments, only 19 (35%) of them were confirmed to be significant when individual typing was performed. This indicates the importance of performing

TABLE 3. Nineteen Positive Microsatellite Markers From Individual Typing

Markers	Cytobands	No. of Alleles	Positive Alleles	Allele Frequencies		*P*		Odds Ratio	95% CI	Nearest Gene Name
				Case	Control	2×2	2×*m*			
HUMUT617	6q27	5	1	0.155	0.215	0.00287	0.02289	1.54	1.08 to 2.21	SMOC2
D2S0226i	2q35	16	1	0.096	0.058	0.00523	0.12651	1.73	1.18 to 2.55	XRCC5
D17S0287i	17q21.33	8	1	0.390	0.323	0.00746	0.08458	1.34	1.09 to 1.65	CROP
D17S0351i	17q24.3	10	4	0.146	0.198	0.00812	0.00229	0.69	0.53 to 0.90	KCNJ16
D19S0134i	19p13.2	28	2	0.070	0.040	0.01212	0.06104	1.80	1.14 to 2.84	ZNF358
D2S0208i	2q11.2	9	1	0.224	0.174	0.01468	0.06679	1.38	1.07 to 1.77	CHST10
D13S0183i	13q31.3	11	2	0.230	0.179	0.01545	0.13986	1.37	1.07 to 1.76	Unknown
D4S0818i	4p16.1	17	1	0.112	0.153	0.01950	0.35114	0.70	0.52 to 0.94	SORCS2
D2S0949i	2p25.1	11	2	0.105	0.070	0.02343	0.09465	1.54	1.08 to 2.21	LPIN1
D20S885	20p11.23	17	1	0.123	0.165	0.02403	0.55390	0.71	0.53 to 0.95	Unknown
D10S0517i	10q26.13	15	1	0.223	0.177	0.02542	0.10058	1.33	1.04 to 1.72	TACC2
D17S0231i	17p13.1	8	1	0.615	0.558	0.02608	0.18437	1.26	1.03 to 1.54	Unknown
D3S0865i	3p21.31	13	1	0.245	0.199	0.03162	0.27624	1.31	1.03 to 1.66	XCR1
D3S1129i	3p22.1	26	1	0.149	0.112	0.03273	0.43997	1.39	1.03 to 1.88	Unknown
D3S0046i	3p26.1	19	1	0.001	0.010	0.03862	0.59448	0.13	0.02 to 0.73	GRM7
D17S790	17q22	14	1	0.026	0.047	0.04028	0.20889	0.54	0.31 to 0.94	Unknown
D18S0390i	18q22.1	17	2	0.140	0.179	0.04227	0.11569	0.75	0.57 to 0.98	Unknown
G09023	13q33.3	8	1	0.204	0.249	0.04245	0.03990	0.78	0.61 to 0.99	Unknown
D4S0370i	4q34.4	18	1	0.052	0.078	0.04780	0.54383	0.65	0.43 to 0.98	Unknown

individual typing after all of the pooling experiments to validate the pooled frequency estimates.

Essential Hypertension Susceptibility Genes

We have identified 19 MS loci associated with essential hypertension and compared our findings with those of 6 previous large-scale genome-wide studies that are summarized in Table 4. The loci of linkage analysis in the previous 9 reports were too wide (≥5 megabases) to identify and speculate about disease susceptibility genes. Three of the 19 identified regions in our study overlapped with a region identified in other races (Table 4). The studies in Table 4, except for the admixture mapping study,[13] were linkage studies and suggest a much broader region than our results. For example, we found that the positive MS locus D2S0949i is located on cytoband 2p25.1, and this finding is in accordance with the admixture mapping results obtained by Zhu et al,[13] who found evidence for linkage with a marker on chromosome 2p25.1. This concordance between 2 different studies suggests that chromosome 2p25.1 contains an unknown candidate gene for essential hypertension. Interestingly, our MS marker is located within the LPIN1 gene sequence (NM 145693.1), and this is a candidate gene for human lipodystrophy, a disease characterized by loss of body fat, fatty liver, hypertriglyceridemia, and insulin resistance. There have been no reports to indicate that LPIN1 is a candidate gene for essential hypertension, but lipin expression is important for metabolic homeostasis.[40] In consideration that hypertension is an associated factor in the metabolic syndrome characterized by obesity, hypertriglyceridemia, and insulin resistance,[41,42] LPIN1 deserves to be studied as a new candidate gene.

Another significant MS marker, HUMUT617 on 6q27, is in the same cytoband position reported by Caulfield et al[14] Our marker was located within the SMOC2 gene sequence (NM_022138) that codes for a modular extracellular calcium-binding protein[43] and a smooth muscle–associated protein upregulated during neointima formation.[44] There have been no previous reports suggesting any connection between SMOC2

TABLE 4. Summary of Genome-Wide Scan Mapping Analyses on Blood Pressure

References	Chromosome	Ethnicity
Zhu et al.[13]	2p25.1, 3q13.31-33, 6q24, 21q21	Admixture
Caulfield et al.[14]	2q24.1, 5q13.1, 6q27, 9q34.11	White
Rao et al.[15]	2p	Blacks and whites
Kardia et al.[16]	No evidence	Blacks and non-Hispanic whites
Thiel et al.[17]	1	Blacks and whites
Ranade et al.[18]	10p	Chinese and Japanese origin
Harrap et al.[19]	1p34.3-1p31, 4q21-28, 16p13.1-16p12, Xp11.4-Xq11	White
Atwood et al.[20]	2p11.2, 2q12.2, 8q24.3, 18q23, 21q22.13	Mexican Americans
Rice et al.[21]	1p22.3-p13.1, 2p11.1-q12.3, 3q13.31-q26.32, 5p15.2-p12, 7q32.1-q36.1, 8q21.11, 10p14, 12p13.33, 14q11.2-q12, 19p13.3, 22q13.1-q13.2	White

Overlapped locations mapped by this study in comparison with previous studies are underlined.

and BP, but this gene may be involved in the progression of atherosclerosis in the aorta.[44]

Perspectives

We performed an association analysis of essential hypertension using a high-density set of polymorphic MS markers with original, multistep methodology. The outcome was a rapid and efficient path to detect genomic susceptibility loci for a highly complex disorder. MS markers basically play a role as location markers for regions containing susceptibility and protective genes. Rarely, MS markers may be the causative variance themselves. The next step is to identify susceptibility and protective genes in the 19 narrow regions by SNP, LD block, and haplotype analysis. It is also important to replicate these results in different subjects, ethnic groups, and a larger number of samples. The future successful accomplishment of such analysis will also open the door to investigating the etiology of other multifactorial disorders, including common diseases such as bronchial asthma, type 2 diabetes mellitus, obesity, arteriosclerosis, schizophrenia, and psoriasis.

BP is influenced by nongenetic factors, such as salt intake. In the present study, because we did not focus on salt-induced hypertension, the amounts of urinary excretion of sodium were not examined. It might be noteworthy to perform studies specializing in genes related to salt-induced hypertension.

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Disclosures

None.

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