tive SNPs, and all positive SNPs are scheduled to be genotyped in a large general population to reconfirm the findings, as five G-protein related SNPs were genotyped in the second population in the present study. Accordingly, in the present study, all results were present without any correction based on HWE.

In summary, the findings of the present study indicate that a systemic multiple candidate gene approach can be used to identify not only susceptibility genes but also susceptibility pathways in which related genes may synergistically collaborate through gene–gene interactions to predispose to hypertension. Our findings suggest that the CD/CV hypothesis can be challenged with numerous combinations of common variants with small effects.

References

1. Gong M, Hubner N: Molecular genetics of human hypertension. *Clin Sci* 2006; **110**: 315–326.
2. Tanira MO, Al Balushi KA: Genetic variations related to hypertension: a review. *J Hum Hypertens* 2005; **19**: 7–19.
3. Marteau J-B, Zaiou M, Siest G, Visvikis-Siest S: Genetic determination of blood pressure regulation. *J Hypertens* 2005; **23**: 2127–2143.
4. Agarwal A, Williams GH, Fisher NDL: Genetics of human hypertension. *Trends Endocrinol Metab* 2005; **16**: 127–133.
5. McBride MW, Graham D, Delles C, Dominiczak AF: Functional genomics in hypertension. *Curr Opin Nephrol Hypertens* 2006; **15**: 145–151.
6. Williams SM, Addy JH, Phillips JA 3rd, *et al*: Combinations of variations in multiple genes are associated with hypertension. *Hypertension* 2000; **36**: 2–6.
7. Williams SM, Ritchie MD, Phillips JA 3rd, *et al*: Multilocus analysis of hypertension: a hierarchical approach. *Hum Hered* 2004; **57**: 28–38.
8. Caulfield M, Munroe P, Pembroke J, *et al*: MRC British Genetics of Hypertension Study. Genome-wide mapping of human loci for essential hypertension. *Lancet* 2003; **361**: 2118–2123.
9. Nebert DW: Polymorphisms in drug-metabolizing enzymes: what is their clinical relevance and why do they exist? *Am J Hum Genet* 1997; **60**: 265–271.
10. Izawa H, Yamada Y, Okada T, Tanaka M, Hirayama H, Yokota M: Prediction of genetic risk for hypertension. *Hypertension* 2003; **41**: 1035–1040.
11. Kokubo Y, Tomoike H, Tanaka C, *et al*: Association of sixty-one non-synonymous polymorphisms in forty-one hypertension candidate genes with blood pressure variation and hypertension. *Hypertens Res* 2006; **29**: 611–619.
12. Kosachunhanun N, Hunt SC, Hopkins PN, *et al*: Genetic determinants of nonmodulating hypertension. *Hypertension* 2003; **42**: 901–908.
13. Agachan B, Isbir T, Yilmaz H, Akoglu E: Angiotensin converting enzyme I/D, angiotensinogen T174M-M235T and angiotensin II type 1 receptor A1166C gene polymorphisms in Turkish hypertensive patients. *Exp Mol Med* 2003; **35**: 545–549.
14. Vasku A, Soucek M, Znojil V, *et al*: Angiotensin I–converting enzyme and angiotensinogen gene interaction and prediction of essential hypertension. *Kidney Int* 1998; **53**: 1479–1482.
15. Giner V, Poch E, Bragulat E, *et al*: Renin-angiotensin system genetic polymorphism and salt sensitivity in essential hypertension. *Hypertension* 2000; **35**: 512–517.
16. Tsai CT, Fallin D, Chiang FT, *et al*: Angiotensinogen gene haplotype and hypertension: interaction with ACE gene I allele. *Hypertension* 2003; **41**: 9–15.
17. Dominiczak AF, Graham D, McBride MW, *et al*: Corcoran Lecture. Cardiovascular genomics and oxidative stress. *Hypertension* 2005; **45**: 636–642.
18. Haga H, Yamada R, Ohnishi Y, Nakamura Y, Tanaka T: Gene-based SNP discovery as part of the Japanese Millennium Genome Project: identification of 190,562 genetic variations in the human genome. Single-nucleotide polymorphism. *J Hum Genet* 2002; **47**: 605–610.
19. Tamaki S, Nakamura Y, Tabara Y, *et al*: Relationship between metabolic syndrome and Trp64Arg polymorphism of the β-adrenergic receptor gene in a general sample: the Shigaraki study. *Hypertens Res* 2006; **29**: 891–896.
20. Sugimoto K, Katsuya T, Ohkubo T, *et al*: Association between angiotensin II type 1 receptor gene polymorphism and essential hypertension: the Ohasama Study. *Hypertens Res* 2004; **27**: 551–556.
21. Tabara Y, Tachibana-Iimori R, Yamamoto M, *et al*: Hypotension associated with prone body position: a possible overlooked postural hypotension. *Hypertens Res* 2005; **28**: 741–746.
22. Yamamoto M, Jin JJ, Wu Z, *et al*: Interaction between serotonin 2A receptor and endothelin-1 variants in association with hypertension in Japanese. *Hypertens Res* 2006; **29**: 227–232.
23. Hirakawa M, Tanaka T, Hashimoto Y, Kuroda M, Takagi T, Nakamura Y: JSNP: a database of common gene variations in the Japanese population. *Nucleic Acids Res* 2002; **30**: 158–162.
24. Kittler R, Stoneking M, Kayser M: A whole genome amplification method to generate long fragments from low quantities of genomic DNA. *Anal Biochem* 2002; **300**: 237–244.
25. Tachibana-Iimori R, Tabara Y, Kusuhara H, *et al*: Effect of genetic polymorphism of OATP-C (SLCO1B1) on lipid-lowering response to HMG-CoA reductase inhibitors. *Drug Metab Pharmacokinet* 2004; **19**: 375–380.
26. Bannai M, Higuchi K, Akesaka T, *et al*: Single-nucleotide-polymorphism genotyping for whole-genome-amplified samples using automated fluorescence correlation spectroscopy. *Anal Biochem* 2004; **327**: 215–221.
27. Ritchie MD, Hahn LW, Roodi N, *et al*: Multifactor-dimensionality reduction reveals high-order interactions among estrogen-metabolism genes in sporadic breast cancer. *Am J Hum Genet* 2001; **69**: 138–147.
28. Harrap SB: Where are all the blood-pressure genes? *Lancet* 2003; **361**: 2149–2151.
29. Doris PA: Hypertension genetics, single nucleotide polymorphisms, and the common disease: common variant hypothesis. *Hypertension* 2002; **39**: 323–331.
30. Liu PY, Zhang YY, Lu Y, *et al*: A survey of haplotype variants at several disease candidate genes: the importance of rare variants for complex diseases. *J Med Genet* 2005; **42**:

221–227.

31. Cohen JC, Kiss RS, Pertsemlidis A, Marcel YL, McPherson R, Hobbs HH: Multiple rare alleles contribute to low plasma levels of HDL cholesterol. *Science* 2004; **305**: 869–872.
32. Kostenis E, Waelbroeck M, Milligan G: Techniques: promiscuous Galpha proteins in basic research and drug discovery. *Trends Pharmacol Sci* 2005; **26**: 595–602.
33. Cabrera-Vera TM, Vanhauwe J, Thomas TO, *et al*: Insights into G protein structure, function, and regulation. *Endocr Rev* 2003; **24**: 765–781.
34. Riddle EL, Schwartzman RA, Bond M, Insel PA: Multitasking RGS proteins in the heart: the next therapeutic target? *Circ Res* 2005; **96**: 401–411.
35. Nunn C, Mao H, Chidiac P, Albert PR: RGS17/RGSZ2 and the RZ/A family of regulators of G-protein signaling. *Semin Cell Dev Biol* 2006; **17**: 390–399.
36. Ho MK, Yung LY, Chan JS, Chan JH, Wong CS, Wong YH: $G\alpha_{14}$ links a variety of G_i- and G_s-coupled receptors to the stimulation of phospholipase C. *Br J Pharmacol* 2001; **132**: 1431–1440.
37. Wang Y, Ho G, Zhang JJ, *et al*: Regulator of G protein signaling Z1 (RGSZ1) interacts with $G\alpha_i$ subunits and regulates $G\alpha_i$-mediated cell signaling. *J Biol Chem* 2002; **277**: 48325–48332.
38. Cho YM, Ritchie MD, Moore JH, *et al*: Multifactor-dimensionality reduction shows a two-locus interaction associated with type 2 diabetes mellitus. *Diabetologia* 2004; **47**: 549–554.
39. Tsai CT, Lai LP, Lin JL, *et al*: Renin-angiotensin system gene polymorphisms and atrial fibrillation. *Circulation* 2004; **109**: 1640–1646.
40. Colhoun HM, McKeigue PM, Davey Smith G: Problems of reporting genetic associations with complex outcomes. *Lancet* 2003; **361**: 865–872.
41. Mayeux R: Mapping the new frontier: complex genetic disorders. *J Clin Invest* 2005; **115**: 1404–1407.
42. Wacholder S, Chanock S, Garcia-Closas M, El Ghormli L, Rothman N: Assessing the probability that a positive report is false: an approach for molecular epidemiology studies. *J Natl Cancer Inst* 2004; **96**: 434–442.
43. Newton-Cheh C, Hirschohorn JN: Genetic association studies of complex traits: design and analysis issues. *Mutat Res* 2005; **573**: 54–69.
44. Rudan I, Smolej-Narancic N, Campbell H, *et al*: Inbreeding and the genetic complexity of human hypertension. *Genetics* 2003; **163**: 1011–1021.
45. Trikalinos TA, Salanti G, Khoury MJ, Ioannidis JP: Impact of violations and deviations in Hardy-Weinberg equilibrium on postulated gene-disease associations. *Am J Epidemiol* 2006; **163**: 300–309.

HJH 201067

Association of *TNFRSF4* gene polymorphisms with essential hypertension

Yoichi Mashimo[a], Yoichi Suzuki[a], Kazuko Hatori[a], Yasuharu Tabara[b], Tetsuro Miki[c], Katsushi Tokunaga[d], Tomohiro Katsuya[e], Toshio Ogihara[e], Michiko Yamada[f], Norio Takahashi[g], Yoshio Makita[h], Tomohiro Nakayama[i], Masayoshi Soma[j], Nobuhito Hirawa[k], Satoshi Umemura[k], Takayoshi Ohkubo[l], Yutaka Imai[m] and Akira Hata[a]

Essential hypertension is a complex disorder that results from the interaction of a number of susceptibility genes and environmental factors. The TNFRSF4 (tumor necrosis factor receptor superfamily, member 4) gene was one of the genes that showed altered renal expression in long-term salt loading in mice. Moreover, association of the TNFRSF4 and TNFSF4 (tumor necrosis factor (ligand) superfamily, member 4) genes with myocardial infarction was recently reported. Since essential hypertension is a well-known risk factor for myocardial infarction, we hypothesized that TNFRSF4 could be a susceptibility gene for essential hypertension. We performed a case–control study of TNFRSF4 in two independent population. Extensive investigation of single nucleotide polymorphisms of the entire gene suggested that it resided in one linkage disequilibrium block, and four single nucleotide polymorphisms in the 5′ flanking region sufficiently represented major haplotypes. In the combined population, the frequency of the most frequent haplotype, C-C-A-A, was significantly lower ($P = 8.07 \times 10^{-5}$) and that of the second most frequent haplotype, C-T-G-A, was significantly higher ($P = 6.07 \times 10^{-4}$) in hypertensive subjects than in control subjects. This difference was observed only in female patients. The C-T-G-A haplotype showed a lower promoter activity than other haplotypes, suggesting a relationship with disease susceptibility. Our results suggest that TNFRSF4 is a female-specific susceptible gene for essential hypertension. *J Hypertens* 26:000–000 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Journal of Hypertension 2008, 26:000–000

Keywords: association studies, essential, haplotype, hypertension, single nucleotide polymorphisms, *TNFRSF4* gene

[a]Department of Public Health, Graduate School of Medicine, Chiba University, Chiba, [b]Department of Medical Genomics, [c]Department of Geriatric Medicine, School of Medicine, Ehime University, Ehime, [d]Department of Human Genetics, Graduate School of Medicine, University of Tokyo, Tokyo, [e]Department of Geriatric Medicine, Graduate School of Medicine, Osaka University, Osaka, [f]Department of Clinical Studies, [g]Department of Genetics, Radiation Effects Research Foundation, Hiroshima, [h]Department of Pediatrics, Asahikawa Medical College, Asahikawa, [i]Division of Molecular Diagnostics, Advanced Medical Research Center, [j]Division of Nephrology and Endocrinology, Department of Medicine, Nihon University School of Medicine, Tokyo, [k]Second Department of Internal Medicine, Graduate School of Medicine, Yokohama City University, Kanagawa, [l]Department of Planning for Drug Development and Clinical Evaluation and [m]Department of Clinical Pharmacology and Therapeutics, Graduate School of Pharmaceutical Sciences and Medicine, Tohoku University, Sendai, Japan

Correspondence to Akira Hata MD, PhD, Department of Public Health, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan
Tel: +81 43 2262067; fax: +81 43 2262070; e-mail: ahata@faculty.chiba-u.jp

Received ?? ?? Revised ?? ??
Accepted ?? ??

AQ1

Introduction

Hypertension affects more than 25% of the adult population worldwide [1]. Essential hypertension (EH) accounts for more than 90% of hypertension cases and is a multifactorial disorder resulting from the interaction of a number of susceptibility genes and environmental factors. It is estimated that the genetic contribution to blood pressure variation ranges from 30 to 50% [2]. Identification of susceptibility genes for hypertension would provide a clue to the pathophysiology of the disease.

Several approaches exists for genetic causes of EH: candidate–gene linkage studies, genome–scanning linkage studies, candidate–gene association studies, genetic studies in animal models, and gene expression profiling in animal models [3]. Each approach has its own strengths and weaknesses, and some argue that integration of the approaches is a more efficient way forward [4]. The Millennium Genome Project for Hypertension in Japan has adopted the candidate–gene association strategy because of its relatively higher statistical power and convenience of collecting samples [5]. Candidate genes are selected on the basis of the accumulation of experimental evidence (expression profiling in animal models) and information in the literature. As a first step in this project, we performed DNA microarray experiments in mice to screen genes whose renal expression was changed by long-term salt loading, because genes that showed salt sensitivity were considered to be candidate genes for EH. The results showed that more than 300 genes were either

0263-6352 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins

downregulated or upregulated. For the genetic association study, from these 300 genes, we nominated 121 that had been reported in the literature as candidate genes. To date, 70 genes have been screened, 10 of which showed significant association with EH on haplotype-based analysis. Three of these 10 genes were positive in both the expression profiling and genetic association studies. The tumor necrosis factor receptor superfamily, member 4 (*TNFRSF4*) gene was one of the three.

TNFRSF4 (OX40) is a member of the tumor necrosis factor receptor (TNFR) superfamily, and is primarily expressed as a transmembrane protein on activated $CD4^+$ T cells after antigen recognition [6–9]. Tumor necrosis factor (ligand) superfamily, member 4 (TNFSF4, also called OX40L) [10], the ligand for TNFRSF4 on activated $CD4^+$ T cells, is expressed on antigen-presenting cells (APCs) including activated B cells, macrophages, and dendritic cells, as well as on endothelial cells and some activated T cells [11–14]. The TNFRSF4–TNFSF4 interaction between T cell and APC contributes to proinflammatory T-cell function. In particular, TNFRSF4–TNFSF4 interactions are crucial for the generation of memory $CD4^+$ T cells by promoting the survival of effector T cells [15–18]. Thus, it is suggested that the TNFRSF4–TNFSF4 pathway is involved in inflammation and immune response.

T lymphocyte activation involving several receptor–ligand pairs such as TNFRSF4–TNFSF4 is suggested to promote atherosclerosis [12,19,20], which is now considered to be an inflammatory disease [21]. Recently, *TNFSF4* was identified as a susceptibility gene for atherosclerosis and a genetic variation in *TNFSF4* was reported to be associated with myocardial infarction (MI) and severity of coronary artery disease [22]. Genetic variation in *TNFRSF4* was also shown to be associated with MI [23]. These reports suggested that the TNFRSF4–TNFSF4 pathway plays an important role in the pathogenesis of atherosclerosis and MI in humans. It is generally believed that hypertension is one of the major risk factors for atherosclerosis and MI [24]; however, MI and hypertension often coexist, as seen in the SHEEP study cohort in which MI patients were significantly associated with hypertension [25]. Thus, the association between MI and TNFRSF4/TNFSF4 in human subjects may be due to not only atherogenesis but also hypertension itself. We hypothesized that *TNFRSF4* and/or *TNFSF4* were potential candidate genes for EH.

The aim of the present study is to investigate the association between genetic variations of the *TNFRSF4* gene and EH in the Japanese population. We performed a case–control study using two independent population of Japanese patients with EH.

Methods

Study subjects

Initial screening of candidate genes involved 1035 subjects with EH (762 men and 273 women) and 1058 age-matched controls (792 men and 266 women) who were recruited through the study group of the Millennium Genome Project for Hypertension [5]. Six medical institutes took part in the collaborative study and recruited subjects in Japan. Recruitment procedures, case–control criteria, and clinical characteristics are described in detail elsewhere [5].

The clinical characteristics of the subjects included in this study for *TNFRSF4* gene analyses are shown in Tables 1 and 2. Subjects in population 1 were part of the population recruited through the study group of the Millennium Genome Project for Hypertension [5]. Subjects in population 2 were recruited from Ohasama, a cohort in a rural community of northern Japan [26].

Each subject was assigned to one of the blood pressure diagnostic categories defined by the criteria of the 1999 WHO/ISH guidelines for the management of hypertension [27]. Hypertensive (HT) subjects had systolic blood pressure (SBP) of at least 140 mmHg or diastolic blood pressure (DBP) of at least 90 mmHg or were patients currently taking chronic antihypertensive medication. Normotensive (NT) subjects had SBP/DBP lower than 140/90 mmHg and had never been treated with antihypertensive medication. Informed consent was obtained

Table 1 Characteristics of subjects in population 1

	Total subjects			Male subjects			Female subjects		
Parameters	NT	HT	*P*	NT	HT	*P*	NT	HT	*P*
No. of subjects	562	587		301	316		261	271	
Age (years)	61.6 ± 9.2	60.1 ± 11.2	0.011*	59.9 ± 9.0	58.5 ± 11.1	0.083	63.6 ± 9.1	62.0 ± 11.1	0.056
BMI (kg/m^2)	22.2 ± 2.8	23.9 ± 3.3	<0.001*	22.1 ± 2.9	23.8 ± 3.1	<0.001*	22.3 ± 2.7	24.0 ± 3.6	<0.001*
SBP (mmHg)	111.7 ± 8.9	163.7 ± 21.1	<0.001*	111.8 ± 8.8	162.1 ± 18.4	<0.001*	111.5 ± 9.1	166.1 ± 24.4	<0.001*
DBP (mmHg)	68.9 ± 7.3	98.3 ± 14.8	<0.001*	69.4 ± 7.3	98.7 ± 14.0	<0.001*	68.0 ± 7.3	97.6 ± 16.0	<0.001*
TC (mg/dl)	205.5 ± 38.0	207.1 ± 34.9	0.596	195.8 ± 35.7	198.7 ± 33.0	0.294	216.2 ± 37.6	216.0 ± 34.7	0.970
HDL-C (mg/dl)	57.3 ± 15.1	58.3 ± 17.2	0.314	55.3 ± 15.0	56.8 ± 17.5	0.263	59.5 ± 15.0	60.0 ± 16.7	0.697
TG (mg/dl)	123.8 ± 87.4	141.7 ± 84.7	0.003*	132.1 ± 106.2	147.0 ± 94.5	0.133	116.7 ± 67.4	135.4 ± 71.3	0.007*

Values are mean ± SD. BMI, body mass index; DBP, diastolic blood pressure; HDL-C, HDL cholesterol; HT, hypertensive patient; NT, normotensive patient; SBP, systolic blood pressure; TC, total cholesterol; TG, triglyceride. * Difference was statistically significant.

Table 2 Characteristics of subjects in population 2

Parameters	Total subjects			Male subjects			Female subjects		
	NT	HT	*P*	NT	HT	*P*	NT	HT	*P*
No. of subjects	925	732		317	323		608	409	
Age (years)	54.6 ± 11.5	61.6 ± 9.7	<0.001*	55.8 ± 11.1	61.5 ± 10.2	<0.001*	54.0 ± 11.6	61.7 ± 9.3	<0.001*
BMI (kg/m²)	23.4 ± 3.1	24.2 ± 3.3	<0.001*	23.5 ± 3.0	23.6 ± 3.1	0.506	23.4 ± 3.1	24.6 ± 3.4	<0.001*
SBP (mmHg)	123.9 ± 9.8	142.2 ± 12.1	<0.001*	125.4 ± 8.6	143.9 ± 11.4	<0.001*	123.1 ± 10.3	140.8 ± 12.5	<0.001*
DBP (mmHg)	70.3 ± 7.1	80.2 ± 9.1	<0.001*	71.6 ± 6.9	81.9 ± 9.4	<0.001*	69.6 ± 7.2	78.8 ± 8.6	<0.001*
TC (mg/dl)	193.4 ± 34.2	195.0 ± 33.8	0.358	186.4 ± 33.8	183.9 ± 34.0	0.352	197.1 ± 33.9	203.8 ± 31.0	0.001*
HDL-C (mg/dl)	55.3 ± 14.1	53.8 ± 14.6	0.028*	51.2 ± 14.1	52.8 ± 14.4	0.180	57.4 ± 13.7	54.4 ± 14.7	0.001*
TG (mg/dl)	128.9 ± 73.4	142.5 ± 89.8	0.001*	139.1 ± 85.1	146.3 ± 103.0	0.340	123.7 ± 66.1	139.5 ± 77.9	0.001*

Values are mean ± SD. BMI, body mass index; DBP, diastolic blood pressure; HDL-C, HDL cholesterol; HT, hypertensive patient; NT, normotensive patient; SBP, systolic blood pressure; TC, total cholesterol; TG, triglyceride. *Difference was statistically significant.

from each individual as per the protocol approved by each institution's ethics committee.

DNA microarray experiments in mice

In DNA microarray experiments, we used two lines of mice having different numbers of the functional mouse angiotensinogen gene (*Agt*) [28,29], kindly donated by Professor Oliver Smithies (Department of Pathology, University of North Carolina, Chapel Hill, North Carolina, USA). To observe distinct effects by long-term salt loading, *Agt* 2/2 mice (with four wild-type copies of the *Agt* gene) were fed a high-salt diet containing 8% NaCl for 6 months, whereas *Agt* 0/1 mice (with one wild-type copy of the *Agt* gene) were fed a low-salt diet containing 0.3% NaCl. Total RNA was isolated from the kidneys of mice and differences in gene expression were examined using mouse cDNA microarray (Incyte Genomics Inc., Palo Alto, California, USA), which contains 9222 mouse cDNA clones.

Screening of candidate genes

We selected a total of 121 candidate genes (Supplemental Table S1) based on the following criteria: (1) genes reported as candidates in the literature or with functions relevant to the blood pressure regulation and (2) human homologue of genes in which renal expression was changed by long-term salt loading in mice. For an initial screening of these candidate genes, some of the available single nucleotide polymorphisms (SNPs) per gene were selected from the Japanese SNP database (http://snp.ims.u-tokyo.ac.jp/) or dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/) and were genotyped in 1035 patients and 1058 controls using the PCR-SSP-FCS method [30]. Haplotype-based association analyses were performed using SNPAlyze v4.1 Pro software (DYNACOM, Mobara, Japan) based on an expectation/maximization (EM) algorithm. *P* values for overall distribution of haplotypes were calculated by permutation test at 1000 iterations. *P* values less than 0.05 were considered statistically significant.

Screening for polymorphisms in *TNFRSF4*

To identify genetic variants of the human *TNFRSF4* gene, we sequenced all seven exons, the adjacent intronic sequence, 4 kb of the 5′ flanking region, and 1.5 kb of the 3′ flanking region in 32 control subjects. Nineteen primer sets were designed on the basis of the *TNFRSF4* genomic and mRNA sequences from the GenBank database (accession numbers NT_004350.18 and NM_003327, respectively). All polymerase chain reaction (PCR) products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit and an ABI PRIZM 3100 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). The sequences were analyzed and polymorphisms identified using the Genetyx program (Genetyx Corp., Tokyo, Japan).

Genotyping of polymorphisms in *TNFRSF4*

Genotyping of four SNPs in the *TNFRSF4* gene (P1: −3948C>T, P2: −3606C>T, P8: −1725A>G and P12: −530A>G) was performed using either the TaqMan allele-specific amplification (TaqMan-ASA) method [31] or the Custom TaqMan Genomic Assays kit (Applied Biosystems). In the TaqMan-ASA method, specific primers were designed on the basis of the *TNFRSF4* genomic sequence from the GenBank database (accession number NT_004350.18). The primer sequences are shown in Table 3. The PCR mixture for the TaqMan-ASA method contained 5 μl of 2× TaqMan Universal Mix (Applied Biosystems), 0.4 μmol/l of each PCR primer, 0.12 μmol/l of TaqMan probe, and 5 ng of template DNA in a final volume of 10 μl. The samples were analyzed with an ABI PRIZM 7000 Sequence Detection system (Applied Biosystems). The thermoprofiles were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

Luciferase assay

TNFRSF4 reporter constructs of 3970 bp (nt −3968 to +2) were created by means of PCR amplification of genomic DNA from homozygous subjects who had alternative haplotypes with the use of following primers: forward, 5′-GGGGTACCGTGCCACATGGCTGGAATTTAC-3′ (including *KpnI* site) and reverse, 5′-TCTAGCTAGC GTCTCTGCTGTCGCCAGAGTC-3′ (including *NheI* site). Amplicons of three haplotypes (Pr-H1, Pr-H2, and Pr-H5) were cloned into the pGL4.10[luc2] vector

Table 3 Primer sequence (5′ → 3′) for TaqMan-ASA genotyping

SNP	Allele-specific primer	Common primer	TaqMan probe[a]
P1	CACATGGCTGGAATTTACCATC CACATGGCTGGAATTTACCTCT	CTCAGCAGTGGGAGAAAAACAA	CCTCTGAAGCGTTTTCACTGGTATCATGTGT
P2	GTCGCCTTTCCCCCTCCG GTCGCCTTTCCCCCTCCA	GCTGCAGCCAATAGGCACCTT	AATAGCCACTTCGTGCGGCTGG
P8	GTCACAGGTCCAAGAAAGCCGT GTCACAGGTCCAAGAAAGCCGC	GCAGGCTGCCTTACAGACCTT	TGAGCTCTGGGTCAGTGTCCA
P12	GGTCAGGAGTTCAAGACCAGTGT GGTCAGGAGTTCAAGACCAGTTC	CCACGCCCGAATAATTTTTG	AGTAGAGACGGGATTTCGCCATGTTAGC

[a] TaqMan probes contained a 5′ FAM (6-carboxyfluorescein) reporter fluorophore and a 3′ TAMRA (6-carboxytetramethylrhodamine) quencher.

(Promega, Madison, Wisconsin, USA). Promoter constructs that contained one polymorphic change (Pr-P2-T, Pr-P3-T, Pr-P4-del, Pr-P6-G, Pr-P8-G, Pr-P9-G, Pr-P10-T, and Pr-P11-G) were created by site-directed mutagenesis carried out in the Pr-H1 plasmid using the GeneEditor *in vitro* site-directed mutagenesis system (Promega). All constructs were verified by sequencing. COS-7 cells (monkey kidney, SV40 T antigen transformed) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. HEK293 cells (human embryonic kidney) were cultured in minimum essential medium supplemented with 2 mmol/l L-glutamine, 1% nonessential amino acids, 10% fetal bovine serum, and antibiotics. Cells in 12-well plates at 50–70% confluence were transfected with 500 ng of each construct and 10 ng of pGL4.74[hRluc/TK] *Renilla* luciferase vector (Promega) as an internal control for transfection efficiency, using 1.5 μl of FuGENE 6 transfection reagent (Roche Diagnostics, Basel, Switzerland). After 24 h of transfection, the cells were harvested, and firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay System and a TD-20/20 luminometer (Promega). Each experiment was repeated five or six times, and each sample was studied in triplicate.

Statistical analysis

Haploview version 3.32 (http://www.broad.mit.edu/mpg/haploview/index.php) was used to analyze and visualize the linkage disequilibrium (LD) and haplotypic patterns. Hardy-Weinberg equilibrium was assessed by χ^2 analysis. Overall distributions of the genotypes or alleles were analyzed by χ^2 analysis using 2×3 or 2×2 contingency tables between NT controls and HT patients. Haplotype frequencies were estimated using SNPAlyze v4.1 Pro software. The distributions of each haplotype between NT controls and HT patients were calculated both by χ^2 tests of one haplotype against the others (haplotype-wise test) and by permutation tests with 1000 iterations using SNPAlyze software. We calculated odds ratios (ORs) with 95% confidence intervals (CIs) using logistic regression analyses with or without clinical covariates (age, BMI, total cholesterol, high-density lipoprotein cholesterol, and triglyceride). To estimate the contribution of the gene to the total variance of blood pressure, the variance component procedure with the analysis of variance (ANOVA) type III variance estimates was used. Comparisons in reporter assays were performed using Student's *t*-test or ANOVA. All statistical analyses were performed with SPSS software (SPSS Japan Inc., Tokyo, Japan) unless otherwise stated. *P* values less than 0.05 were considered statistically significant.

Results

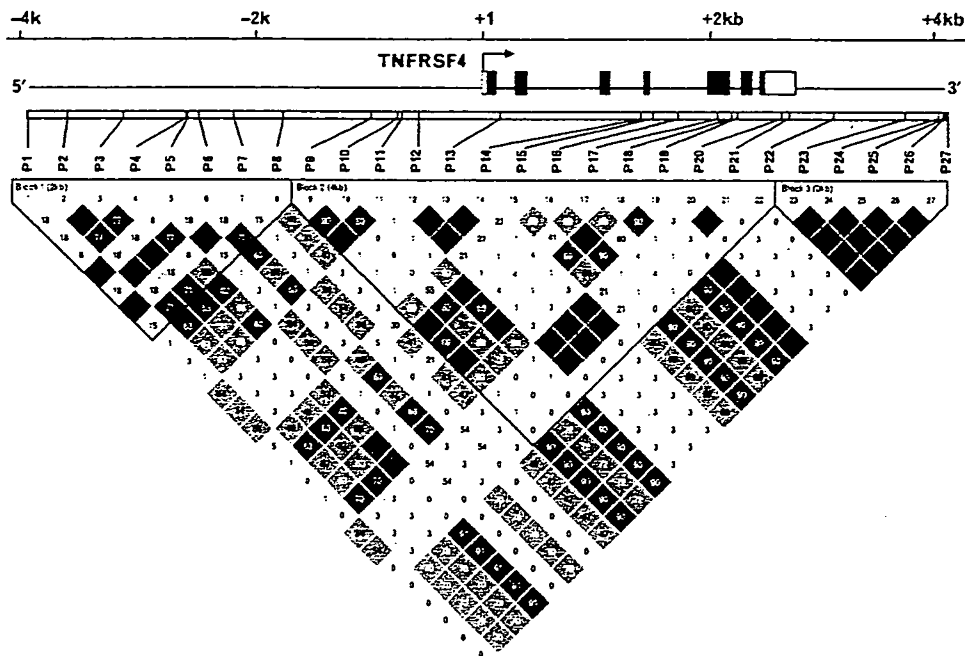
DNA microarray experiments in mice

We used cDNA microarray analyses to compare the expression profiles of 9222 genes in the kidneys of *Agt* 2/2 mice (with four wild-type copies of the *Agt* gene) with a high-salt diet versus those of *Agt* 0/1 mice (with one wild-type copy of the *Agt* gene) with a low-salt diet. Differential expression values greater than 1.3 based on internal quality control data are summarized in Supplemental Tables S2 and S3. We found that 119 genes were downregulated in the kidneys of *Agt* 2/2 mice by 1.3–3.1-fold compared with *Agt* 0/1 mice and 192 genes were upregulated by 1.3–1.9-fold. Murine *TNFRSF4* gene (*Tnfrsf4*) was the gene downregulated 1.3-fold.

Screening of candidate genes by haplotype association study

We selected a total of 121 candidate genes (Supplemental Table S1) on the basis of the following criteria: (1) genes reported as candidates in the literature or with possible involvement of blood pressure regulation and (2) human homologue of genes in which renal expression was changed by long-term salt loading in mice. We excluded genes whose genotype data were not available due to the following reasons: no SNP data was available in the databases; minor allele frequencies of SNPs in Japanese were too low (<5%); or the genotyping of some SNPs was difficult. So far, 191 SNPs in 70 genes have been successfully genotyped for genetic association tests, and the genotyping of only a single SNP was completed in 8 genes. A haplotype-based association test was performed in 62 genes and a single SNP association study in 8 genes. *P* values for difference in overall distribution of the haplotype or genotype frequencies between normotension and hypertension in total (men + women), male, and female subjects are shown in Supplemental Table S4. Significant *P* values were observed for 10 genes;

Fig. 1



Haplotype block structure of the *TNFRSF4* gene. (Top) Organization of the *TNFRSF4* gene. Exons are indicated by boxes (black, coding sequences; white, untranslated sequences). (Bottom) Linkage disequilibrium structure of polymorphisms across the *TNFRSF4* gene region using data from 32 Japanese controls. Haplotype blocks were defined by the solid spine of LD method in Haploview. The number in each cell represents the LD parameter r^2 (100×), blank cells denote $r^2 = 1$. Each cell is painted with graduated color relative to the strength of LD between markers, which is defined by the r^2 value.

Aquaporin-2 (*AQP2*), *Estrogen receptor 2* (*ESR2*), *Glycogen synthase 1* (*GYS1*), *Kallikrein 1* (*KLK1*), *Nephrin* (*NPHN*), *Solute carrier family 1 (glial high affinity glutamate transporter), member 2* (*SLC1A2*), *Solute carrier family 9 (sodium/hydrogen exchanger), isoform 3* (*SLC9A3*), *Steroidogenic acute regulatory protein* (*STAR*), *Syntaxin binding protein 1* (*STXBP1*), and *TNFRSF4*. Three genes (*STAR*, *STXBP1*, and *TNFRSF4*) are the human homologues to the mouse genes that showed changes in renal expression in the salt-loading experiment. The *P* value for overall distribution of the haplotype of *TNFRSF4* was significant only in female subjects.

Identification of polymorphisms in TNFRSF4

We searched for polymorphisms in the *TNFRSF4* gene, including 4 kb of the 5′ flanking region and 1.5 kb of the 3′ flanking region. By direct sequencing in 32 Japanese individuals, a total of 44 polymorphisms were identified; 20 in the 5′ flanking region, 4 in exons, 7 in introns, and 13 in the 3′ flanking region. Of those, 27 polymorphisms (P1–P27) with minor allele frequencies (MAF) 5% of higher (in 32 DNA samples) are presented in Table 4. A graphical overview of the structure of the human *TNFRSF4* gene showing the location of the 27 polymorphisms identified in this study is shown in Fig. 1. Pairwise LD measuring r^2 between polymorphisms and defined haplotype block structures in this region was evaluated using the solid spine of LD method in Haploview (Fig. 1). Three haplotype blocks (blocks 1, 2, and 3) were defined in the *TNFRSF4* gene region with this method. Blocks 1 and 2 appear to be separated because P8 showed low LD to other polymorphisms and blocks 2 and 3 were separated by P22 for the same reason. Strong LDs, however, were observed among certain blocks, such as between P4 and P27 ($r^2 = 0.91$). In addition, multiallelic D′ values between these blocks were high (0.86 between blocks 1 and 2; 1.0 between blocks 2 and 3). Thus, we decided to handle an entire gene region as one block, which could be analyzed by tag SNPs from the entire region. Four SNPs in the 5′ flanking region (P1: −3948C>T, P2: −3606C>T, P8: −1725A>G, and P12: −530A>G) were employed for further analysis. The four SNP haplotypes constructed from these SNPs covered more than 85% of haplotype diversity of the entire *TNFRSF4* gene when P22 was not included for analysis.

Table 4 Polymorphisms with minor allele frequencies 5% or higher detected in the *TNFRSF4* genomic region in 32 Japanese controls

Name	Polymorphism[a]	Location	Amino acid change	MAF[b]	dbSNP ID	JSNP ID
P1	−3943C>T	5′ Flanking		0.06		
P2	−3601C>T	5′ Flanking		0.27	rs12036216	
P3	−3119G>T	5′ Flanking		0.27	rs11721	
P4	−2577delA	5′ Flanking		0.22		
P5	−2568C>G	5′ Flanking		0.06		
P6	−2461C>G	5′ Flanking		0.27		
P7	−2167C>T	5′ Flanking		0.06		
P8	−1720A>G	5′ Flanking		0.30	rs3813201	JST-IMS173304
P9	−936A>G	5′ Flanking		0.19	rs34115518	
P10	−699C>T	5′ Flanking		0.16	rs35339498	
P11	−669C>G	5′ Flanking		0.19	rs35659545	
P12	−525A>G	5′ Flanking		0.11	rs35107976	
P13	150 + 47G>C	Intron1		0.11	rs35737009	
P14	376-16C>G	Intron3		0.11	rs34108055	
P15	442 + 32ins35bp	Intron4		0.25		
P16	442 + 248C>T	Intron4		0.19	rs9661697	
P17	539G>A	Exon5	Glu178Glu	0.25	rs17568	
P18	639 + 25C>T	Intron5		0.19	rs2298212	JST-IMS053053
P19	640-31T>G	Intron5		0.20	rs2298211	JST-IMS053052
P20	921C>T	Exon7 (3′ UTR)		0.11	rs2298210	JST-IMS053051
P21	989C>G	Exon7 (3′ UTR)		0.11	rs2298209	JST-IMS053050
P22	1067 + 308G>A	3′ Flanking		0.08	rs2298208	JST-IMS053049
P23	1067 + 941G>C	3′ Flanking		0.20	rs34067070	
P24	1067 + 1224delTT	3′ Flanking		0.20		
P25	1067 + 1240G>C	3′ Flanking		0.20	rs34279802	
P26	1067 + 1266T>C	3′ Flanking		0.20	rs35916760	
P27	1067 + 1296C>T	3′ Flanking		0.20	rs36057244	

[a] Numbering according to the cDNA sequence of *TNFRSF4* (accession number NM_003327). [b] Minor allele frequency (MAF) on the basis of the sequencing of 32 DNA samples.

Case–control study for *TNFRSF4* polymorphisms

The clinical characteristics of the NT and HT subjects in population 1 are summarized in Table 1. Difference in age between the NT and HT subjects was significant when men and women were jointly compared ($P = 0.011$), whereas it was not significant when men and women were separately compared.

In population 1, four SNPs (P1, P2, P8, and P12) were genotyped in 562 NT controls and 587 HT patients. All

Table 5 Genotype and allele frequencies among normotensive (NT) and hypertensive (HT) subjects in population 1

		Total subjects			Male subjects			Female subjects		
	Genotype	NT ($n = 562$)	HT ($n = 587$)	P[a]	NT ($n = 301$)	HT ($n = 316$)	P[a]	NT ($n = 261$)	HT ($n = 271$)	P[a]
P1	CC	448 (0.799)	455 (0.776)		238 (0.793)	245 (0.775)		210 (0.805)	210 (0.778)	
	CT	109 (0.194)	124 (0.212)		60 (0.200)	67 (0.212)		49 (0.188)	57 (0.211)	
	TT	4 (0.007)	7 (0.012)	0.524	2 (0.007)	4 (0.013)	0.691	2 (0.008)	3 (0.011)	0.722
	Allele									
	C	1005 (0.896)	1034 (0.882)		536 (0.893)	557 (0.881)		469 (0.898)	477 (0.883)	
	T	117 (0.104)	138 (0.118)	0.305	64 (0.107)	75 (0.119)	0.506	53 (0.102)	63 (0.117)	0.429
P2	CC	324 (0.578)	319 (0.544)		166 (0.553)	170 (0.538)		158 (0.605)	149 (0.552)	
	CT	208 (0.371)	220 (0.375)		121 (0.403)	117 (0.370)		87 (0.333)	103 (0.381)	
	TT	29 (0.052)	47 (0.080)	0.129	13 (0.043)	29 (0.092)	0.055	16 (0.061)	18 (0.067)	0.455
	Allele									
	C	856 (0.763)	858 (0.732)		453 (0.755)	457 (0.723)		403 (0.772)	401 (0.743)	
	T	266 (0.237)	314 (0.268)	0.089	147 (0.245)	175 (0.277)	0.203	119 (0.228)	139 (0.257)	0.263
P8	AA	284 (0.506)	280 (0.478)		144 (0.480)	154 (0.487)		140 (0.536)	126 (0.467)	
	AG	238 (0.424)	248 (0.423)		137 (0.457)	125 (0.396)		101 (0.387)	123 (0.456)	
	GG	39 (0.070)	58 (0.099)	0.182	19 (0.063)	37 (0.117)	0.044	20 (0.077)	21 (0.078)	0.250
	Allele									
	A	806 (0.718)	808 (0.689)		425 (0.708)	433 (0.685)		381 (0.730)	375 (0.694)	
	G	316 (0.282)	364 (0.311)	0.129	175 (0.292)	199 (0.315)	0.376	141 (0.270)	165 (0.306)	0.202
P12	AA	401 (0.716)	393 (0.671)		209 (0.699)	215 (0.680)		192 (0.736)	178 (0.659)	
	AG	148 (0.264)	175 (0.299)		84 (0.281)	88 (0.278)		64 (0.245)	87 (0.322)	
	GG	11 (0.020)	18 (0.031)	0.179	6 (0.020)	13 (0.041)	0.318	5 (0.019)	5 (0.019)	0.144
	Allele									
	A	950 (0.848)	961 (0.820)		502 (0.839)	518 (0.820)		448 (0.858)	443 (0.820)	
	G	170 (0.152)	211 (0.180)	0.069	96 (0.161)	114 (0.180)	0.355	74 (0.142)	97 (0.180)	0.093

[a] Significant P value after Bonferroni's correction for four loci is 0.0125 (0.05/4).

Table 6 Four SNP haplotypes (P1, P2, P8, and P12) frequency among normotensive (NT) and hypertensive (HT) subjects in population 1

		Male subjects				Female subjects			
	Haplotype[a]	NT ($n = 298$)	HT ($n = 316$)	P^b	Permutation P	NT ($n = 261$)	HT ($n = 270$)	P^b	Permutation P
H1	C-C-A-A	404 (0.677)	413 (0.653)	0.371	0.363	376 (0.720)	356 (0.659)	0.031	0.021*
H2	C-T-G-A	81 (0.136)	96 (0.152)	0.419	0.420	60 (0.116)	75 (0.138)	0.267	0.259
H3	T-T-G-G	63 (0.106)	73 (0.115)	0.584	0.559	52 (0.099)	62 (0.115)	0.405	0.402
H4	C-C-G-G	16 (0.026)	21 (0.033)	0.484	0.470	17 (0.033)	14 (0.027)	0.578	0.617
H5	C-C-A-G	17 (0.029)	18 (0.029)	0.967	0.958	1 (0.002)	19 (0.036)	6.78×10^{-5}*	<0.001*
H6	C-C-G-A	12 (0.021)	5 (0.008)	0.063	0.066	9 (0.018)	12 (0.021)	0.646	0.682
	Others	3 (0.005)	6 (0.010)			7 (0.013)	2 (0.004)		
Entire distribution				0.722[c]				0.003[c]*	

[a] Four loci are P1, P2, P8, and P12, and six predominant haplotypes are listed; 'others' category includes minor haplotypes with less than 1% frequency. [b] Significant *P* value after Bonferroni's correction for major six haplotypes is 0.0083 (0.05/6). [c] *P* value for the entire distribution with permutation test. * Difference was statistically significant.

of these SNPs were in Hardy–Weinberg equilibrium in the NT group. Table 5 shows the distribution of genotypic and allelic frequencies of the four SNPs in each group. The overall distribution of genotype and allele did not significantly differ between the HT and NT groups for total, male, or female subjects. The *P* value of χ^2 test for the difference in the genotypic frequency of P8 between male HT and NT groups was 0.044, which was not significant after Bonferroni's correction (multiplied by 4).

We next analyzed the four SNP haplotypes in population 1 (Table 6). Six common haplotypes (H1–H6) covered approximately 99% of the subjects in the HT and NT groups. The frequencies of each haplotype in men did not differ between the HT and NT groups. In women, the frequency of the major C-C-A-A haplotype (H1) of the HT subjects was significantly lower than that of the NT subjects ($P = 0.031$). Multiple logistic regression in women revealed that the association of the H1/H1 diplotype with hypertension remained significant ($P = 0.006$) after adjustment for age, BMI, total cholesterol, high-density lipoprotein cholesterol, and triglyceride. The OR of the H1/H1 diplotype against the others was 0.56 with a 95% CI of 0.37–0.85. The frequency of the minor C-C-A-G haplotype (H5) of the HT subjects was significantly higher than that of the NT subjects ($P = 6.78 \times 10^{-5}$). H5 haplotype was significantly associated with hypertension in a dominant model ($P = 0.004$) after adjustment for the above factors. The OR of the H5/H5 + H5/other diplotype against the others was 6.93 with a 95% CI of 1.88–25.5.

To confirm an association of the four SNP haplotypes in women with EH, we genotyped them using the second case–control population (population 2) comprising 925 NT controls and 732 HT patients. Table 2 presents the clinical features of the NT controls and HT patients in population 2. All genotype results of four SNPs in each group were consistent with Hardy–Weinberg equilibrium. Table 7 shows the distribution of genotypic and allelic frequencies of four SNPs in each group of population 2. The overall distribution of genotype and allele of all four SNPs did not significantly differ between the HT and NT groups for total or male subjects. Among women, however, significant differences were observed in the allelic frequencies of P2 ($P = 0.005$) and the genotypic and allelic frequencies of P8 ($P = 0.005$ and 0.003, respectively) between the HT and NT subjects even after Bonferroni's correction (multiplied by 4). P2 was still significantly associated with hypertension in women in both a dominant ($P = 0.007$) and recessive model ($P = 0.038$) after adjustment for age, BMI, total cholesterol, high-density lipoprotein cholesterol, and triglyceride. The OR of T/T + C/T against C/C (dominant model) was 1.22 with a 95% CI of 1.05–1.40, and the OR of T/T against C/T + C/C (recessive model) was 1.94 with a 95% CI of 1.04–3.62. P22 was also significantly associated with hypertension in women in both a dominant model ($P = 0.011$) and recessive model ($P = 0.002$) after adjustment for the above factors. The OR of G/G + A/G against A/A (dominant model) was 1.20 with a 95% CI of 1.04–1.38, and the OR of G/G against A/G + G/G (recessive model) was 1.49 with a 95% CI of 1.16–1.92.

Table 8 shows the frequency of four SNP haplotypes in population 2. Among women, the HT subjects showed a significantly lower frequency of H1 (C-C-A-A) ($P = 8.48 \times 10^{-4}$) and a significantly higher frequency of H2 (C-T-G-A) ($P = 6.46 \times 10^{-4}$) than the NT subjects, whereas in men no significant difference in frequencies of haplotypes between the HT and NT groups was observed. Multiple logistic regression in women revealed that the association of H1 haplotype with hypertension remained significant in both a dominant ($P = 0.006$) and recessive model ($P = 0.005$) after adjustment for age, BMI, total cholesterol, high-density lipoprotein cholesterol, and triglyceride. The OR of the H1/H1 + H1/other diplotype against the others (dominant model) was 0.52 with a 95% CI of 0.32–0.83, and the OR of the H1/H1 diplotype against the others (recessive model) was 0.67 with a 95% CI of 0.50–0.89. The H2 haplotype was also significantly associated with hypertension in women in a dominant model ($P = 0.001$) after adjustment for the above factors. The OR of the H2/H2 + H2/other diplotype against the others was 1.40 with a 95% CI of 1.18–1.65. In population 2, the frequency of H5

Table 7 Genotype and allele frequencies among normotensive (NT) and hypertensive (HT) subjects in population 2

		Total subjects			Male subjects			Female subjects		
	Genotype	NT ($n=925$)	HT ($n=732$)	P^a	NT ($n=317$)	HT ($n=323$)	P^a	NT ($n=608$)	HT ($n=409$)	P^a
P1	CC	729 (0.792)	573 (0.786)		253 (0.801)	249 (0.778)		476 (0.788)	324 (0.792)	
	CT	181 (0.197)	147 (0.202)		58 (0.184)	66 (0.206)		123 (0.204)	81 (0.198)	
	TT	10 (0.011)	9 (0.012)	0.929	5 (0.016)	5 (0.016)	0.770	5 (0.008)	4 (0.010)	0.949
	Allele									
	C	1639 (0.891)	1293 (0.887)		564 (0.892)	564 (0.881)		1075 (0.890)	729 (0.891)	
	T	201 (0.109)	165 (0.113)	0.721	68 (0.108)	76 (0.119)	0.530	133 (0.110)	89 (0.109)	0.927
P2	CC	550 (0.598)	403 (0.553)		176 (0.555)	182 (0.567)		374 (0.620)	221 (0.542)	
	CT	323 (0.351)	282 (0.387)		118 (0.372)	123 (0.383)		205 (0.340)	159 (0.390)	
	TT	47 (0.051)	44 (0.060)	0.176	23 (0.073)	16 (0.050)	0.488	24 (0.040)	28 (0.069)	0.017
	Allele									
	C	1423 (0.773)	1088 (0.746)		470 (0.741)	487 (0.759)		953 (0.790)	601 (0.737)	
	T	417 (0.227)	370 (0.254)	0.069	164 (0.259)	155 (0.241)	0.477	253 (0.210)	215 (0.263)	0.005*
P8	AA	464 (0.508)	342 (0.472)		146 (0.465)	157 (0.489)		318 (0.530)	185 (0.458)	
	AG	384 (0.420)	316 (0.436)		139 (0.443)	143 (0.445)		245 (0.408)	173 (0.428)	
	GG	66 (0.072)	67 (0.092)	0.189	29 (0.092)	21 (0.065)	0.436	37 (0.062)	46 (0.114)	0.005*
	Allele									
	A	1312 (0.718)	1000 (0.690)		431 (0.686)	457 (0.712)		881 (0.734)	543 (0.672)	
	G	516 (0.282)	450 (0.310)	0.080	197 (0.314)	185 (0.288)	0.321	319 (0.266)	265 (0.328)	0.003*
P12	AA	630 (0.686)	479 (0.659)		214 (0.677)	208 (0.650)		416 (0.691)	271 (0.666)	
	AG	265 (0.289)	220 (0.303)		93 (0.294)	100 (0.313)		172 (0.286)	120 (0.295)	
	GG	23 (0.025)	28 (0.039)	0.213	9 (0.028)	12 (0.038)	0.690	14 (0.023)	16 (0.039)	0.301
	Allele									
	A	1525 (0.831)	1178 (0.810)		521 (0.824)	516 (0.806)		1004 (0.834)	662 (0.813)	
	G	311 (0.169)	276 (0.190)	0.128	111 (0.176)	124 (0.194)	0.405	200 (0.166)	152 (0.187)	0.231

[a] Significant P value after Bonferroni's correction for four loci is 0.0125 (0.05/4). * Difference was statistically significant.

did not significantly differ between the HT and NT groups for women.

Although trends of frequency changes in the H1 and H2 haplotypes among women in the two independent population were the same, the frequency of H2 showed a significant difference not in population 1 but in population 2. This discrepancy could have been caused by difference in the sample size. When we analyzed the differences in frequencies of each haplotype between the HT and NT groups in combined samples of the two population (Table 9), female HT subjects showed a significantly lower frequency of H1 ($P=8.07\times10^{-5}$) and a significantly higher frequency of H2 ($P=6.07\times10^{-4}$) than the NT subjects. The frequency of H5 of female HT subjects was still significantly higher than that of NT subjects ($P=0.003$). No significant difference in haplotype frequencies between male HT and NT groups was observed.

Variance component estimation of TNFRSF4

The variance estimates of the *TNFRSF4* diplotype and the residual in SBP of the control women of population 1 were 5.5 and 79.6, respectively. Therefore, the *TNFRSF4* gene explains 6.5% of the variation of SBP in this group. The values in DBP were 2.8 and 52.1, respectively, with the gene contributing 5.2% of the variation.

Transcriptional effects of polymorphisms in the promoter region

To study transcriptional effects of the polymorphisms, we transfected COS-7 cells and HEK293 cells with promoter constructs containing the haplotypes in the *TNFRSF4* gene (Pr-H1, Pr-H2, and Pr-H5). In COS-7 cells, promoter activity of the Pr-H2 construct was significantly lower than that of the Pr-H1 or Pr-H5 construct (0.89 for Pr-H2/Pr-H1, $P=0.008$ and 0.91 for Pr-H2/Pr-H5, $P=0.026$; Fig. 2a). The same results were observed in HEK293 cells (0.92

Table 8 Four SNP haplotypes (P1, P2, P8, and P12) frequency among normotensive (NT) and hypertensive (HT) subjects in population 2

		Male subjects				Female subjects			
	Haplotype[a]	NT ($n=303$)	HT ($n=299$)	P^b	Permutation P	NT ($n=584$)	HT ($n=388$)	P^b	Permutation P
H1	C-C-A-A	403 (0.665)	403 (0.674)	0.743	0.714	839 (0.718)	502 (0.647)	8.48×10^{-4}*	<0.001*
H2	C-T-G-A	86 (0.142)	75 (0.125)	0.400	0.388	115 (0.098)	116 (0.149)	6.46×10^{-4}*	0.001*
H3	T-T-G-G	66 (0.109)	71 (0.119)	0.592	0.593	125 (0.107)	84 (0.108)	0.939	0.926
H4	C-C-G-G	30 (0.049)	24 (0.040)	0.429	0.443	47 (0.040)	45 (0.058)	0.067	0.074
H5	C-C-A-G	11 (0.018)	20 (0.034)	0.095	0.113	21 (0.018)	18 (0.023)	0.434	0.451
H6	C-C-G-A	8 (0.014)	4 (0.007)	0.265	0.374	15 (0.013)	8 (0.010)	0.606	0.583
	Others	2 (0.003)	1 (0.002)			6 (0.005)	3 (0.004)		
Entire distribution				0.533[c]				0.026[c,*]	

[a] Four loci are P1, P2, P8, and P12, and six predominant haplotypes are listed; 'others' category includes minor haplotypes with less than 1% frequency. [b] Significant P value after Bonferroni's correction for major six haplotypes is 0.0083 (0.05/6). [c] P value for the entire distribution with permutation test. * Difference was statistically significant.

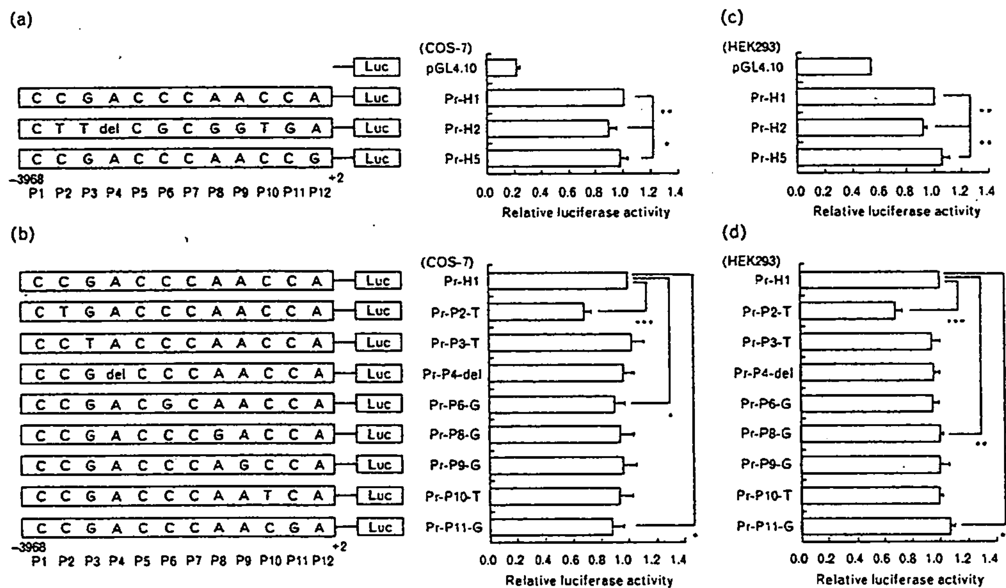
Table 9 Four SNP haplotype (P1, P2, P8, and P12) frequency among normotensive (NT) and hypertensive (HT) subjects in the combined population (population 1 and 2)

	Haplotype[a]	Male subjects			Female subjects		
		NT (n = 602)	HT (n = 615)	P[b]	NT (n = 845)	HT (n = 658)	P[b]
H1	C-C-A-A	806 (0.671)	816 (0.663)	0.682	1215 (0.719)	858 (0.652)	8.07×10^{-5}*
H2	C-T-G-A	167 (0.139)	171 (0.139)	0.989	175 (0.104)	191 (0.145)	6.07×10^{-4}*
H3	T-T-G-G	129 (0.107)	144 (0.117)	0.446	177 (0.104)	146 (0.111)	0.578
H4	C-C-G-G	45 (0.038)	45 (0.036)	0.846	64 (0.038)	60 (0.045)	0.306
H5	C-C-A-G	29 (0.024)	38 (0.031)	0.263	22 (0.013)	37 (0.028)	0.003*
H6	C-C-G-A	21 (0.017)	9 (0.008)	0.033	24 (0.014)	20 (0.015)	0.906
	Others	5 (0.004)	7 (0.006)		13 (0.008)	6 (0.004)	

[a] Four loci are P1, P2, P8, and P12, and six predominant haplotypes are listed; 'others' category includes minor haplotypes with less than 1% frequency. [b] Significant *P* value after Bonferroni's correction for major six haplotypes is 0.0083 (0.05/6). * Difference was statistically significant.

for Pr-H2/Pr-H1, $P = 0.001$ and 0.88 for Pr-H2/Pr-H5, $P = 0.001$; Fig. 2c). There was no significant difference in promoter activity between the Pr-H1 and Pr-H5 constructs in both cells. These results suggest that expression of TNFRSF4 mRNA in cells is lower in individuals who have the H2 haplotype than in cells from individuals who have other types of haplotypes. To clarify the responsible SNP(s) for the lower promoter activity of Pr-H2, we performed an additional assay using a series of promoter constructs that contained only one polymorphic change (Pr-P2-T, Pr-P3-T, Pr-P4-del, Pr-P6-G, Pr-P8-G, Pr-P9-G, Pr-P10-T, and Pr-P11-G). In COS-7 cells, promoter activities of Pr-P2-T, Pr-P6-G, and Pr-P11-G were significantly lower than that of Pr-H1 (0.69 for Pr-P2-T/Pr-H1, $P < 0.0001$, 0.90 for Pr-P6-G/Pr-H1, $P = 0.016$, and 0.88 for Pr-P11-G/Pr-H1, $P = 0.015$; Fig. 2b). In HEK293 cells, as in COS-7 cells, Pr-P2-T showed significantly lower promoter activity when compared with Pr-H1 (0.71 for Pr-P2-T/Pr-H1, $P = 0.0001$; Fig. 2d). The results of other constructs, however, were different: promoter activities of Pr-P8-G and Pr-P11-G were significantly higher than that of Pr-H1 (1.04 for Pr-P8-G/Pr-H1, $P = 0.002$ and 1.10 for

Fig. 2



AQ2 Effect of haplotypes and each polymorphism on the transcriptional activity of the *TNFRSF4* promoter. (a and c) Effect of haplotypes on the transcriptional activity of the *TNFRSF4* promoter. (b and d) Effect of each polymorphism on the transcriptional activity of the *TNFRSF4* promoter. Relative luciferase activities after transient transfection in COS-7 (a and b) and HEK293 (c and d) cell lines are shown. Activities of the Pr-H1 constructs were considered as 100%. Each experiment was conducted in triplicate for each sample, and the results are expressed as mean ± SD for six (COS-7) or five (HEK293) independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Pr-P11-G/Pr-H1, $P = 0.003$; Fig. 2d). Only Pr-P2-T showed consistent change in promoter activity in the two different cell lines. These results suggest that P2 had the largest impact on the decreased promoter activity of the H2 haplotype.

Discussion

The significance of *TNFRSF4* in the pathogenesis of female subjects with EH was indicated in two independent sets of population. Haplotype analysis using four SNPs (P1: −3948C>T, P2: −3606C>T, P8: −1725A>G and P12: −530A>G) in the 5′ upstream region showed that the frequency of H1 (C-C-A-A) was significantly low among female HT patients when compared with female NT controls in both population 1 ($P = 0.031$) and population 2 ($P = 8.48 \times 10^{-4}$). The frequency of H2 (C-G-T-A) of female HT patients was significantly higher than that of female NT controls in population 2 ($P = 6.46 \times 10^{-4}$), but not in population 1. In the combined population, both significantly lower frequency of H1 ($P = 8.07 \times 10^{-5}$) and significantly higher frequency of H2 ($P = 6.07 \times 10^{-4}$) were observed in female HT patients compared with female NT controls. No difference in haplotype frequencies between the HT and NT groups was observed in the male subjects of either the combined or separate population. These results of association of the *TNFRSF4* haplotype with hypertension suggested that the H1 haplotype is a protective allele and that the H2 haplotype is a high-risk allele for EH in women. The promoter activity of the H2 haplotype was significantly lower than that of the H1 and H5 (C-C-A-G) haplotypes. Furthermore, the Pr-P2-T construct showed lower promoter activity than other constructs. Allelic association of P2 (−3606C>T, rs12036216) with female HT patients was significant in population 2 and the combined population (data not shown), but not in population 1. These data suggested that P2 is the responsive SNP that modifies the risk for hypertension in females, although it is possible that unidentified variant(s) in LD with this haplotype have function(s) that influence disease susceptibility. We also observed a significant difference in frequency of the H5 haplotype in the combined population ($P = 0.003$) and in population 1 ($P = 6.78 \times 10^{-5}$), but not in population 2. We, however, could not find any transcriptional effect of H5 haplotype.

The TNFRSF4–TNFSF4 interactions on T lymphocytes enhance proliferation and differentiation of the cells as well as generation and survival of memory $CD4^{+}$ T cells in the process of inflammation and immune response [15–18]. Several inflammatory markers, such as soluble leukocyte adhesion molecules, cytokines, specific growth factors, heat shock proteins, CD40L, and C-reactive protein (CRP), were reported to increase in patients with EH [32–41]. Although the relationship between inflammation and hypertension has not been well established, a growing body of evidence indicates that vascular inflammation may be involved in both the initiation and development of hypertension [42–46]. Sesso *et al.* [46] showed that elevated plasma CRP, a well-known marker of inflammation, was associated with the future development of hypertension in a dose-dependent manner. Furthermore, hypertension has been suggested to trigger inflammation through the increased expression of several mediators, including leukocyte adhesion molecules, chemokines, specific growth factors, heat shock proteins, endothelin-1, and angiotensin [47–54]. Given our findings that variants of the *TNFRSF4* gene, which might affect the inflammatory cascade, were associated with EH among women, it is likely that inflammation may play a role in initiation and/or development of hypertension.

Inflammatory process [21] and T lymphocyte activation [12,19,20] are implicated to be involved in the pathogenesis of atherosclerosis. Thus, alteration(s) in the TNFRSF4–TNFSF4 pathway could influence atherosclerosis formation. Indeed, Wang *et al.* [22] found that polymorphisms of *TNFSF4* are associated with MI in women. Furthermore, a polymorphism in *TNFRSF4* was also reported to be associated with MI [23]. These studies strongly suggested that genes involved in the TNFRSF4–TNFSF4 pathway play a role in the pathogenesis of atherosclerosis and MI, particularly in women.

Our findings combined with those of the studies mentioned above suggested that genetic variations in the TNFRSF4–TNFSF4 pathway may be involved in the pathogenesis of both atherosclerosis and hypertension. So, which comes first, atherosclerosis or hypertension? Hypertension is one of the principal risk factors for atherosclerosis and MI [24], but the exact mechanism underlying the association is not fully understood. Although arterial stiffness, which is a predictor of atherosclerosis [55,56], has been thought to be the result of hypertension rather than its cause, recent studies suggested that arterial stiffness is related to the development of hypertension [57,58]. These data indicated that the relationship between hypertension and arterial stiffness may be bidirectional [59]. Therefore, three different scenarios are possible to explain the results that genetic variations in the TNFRSF4–TNFSF4 pathway are associated with both hypertension and MI: First, inflammation may directly increase arterial stiffness and induce the development of an atherosclerotic lesion, which may lead to the development of hypertension. Second, inflammation may induce hypertension, which may result in increase in arterial stiffness and atherosclerosis. Third, inflammation may promote the development of hypertension and atherosclerosis by different pathways. Although it is not clear whether atherosclerosis is a cause of hypertension, our findings and that of previous studies indicate that the inflammation may be an important part of the link between hypertension and atherosclerosis and cardiovascular events, such as MI.

TNFSF4 is also a potential candidate for a susceptibility gene involved in the pathogenesis of EH in women. We, therefore, examined the putative association between polymorphisms in the *TNFSF4* gene and hypertension in population 1. The allele frequencies of four SNPs (rs1234315, rs3850641, rs1234313, and rs3861950) and its haplotype did not significantly differ between the HT group and the NT group for women (data not shown). In contrast to the case of MI in which susceptibility was affected by variations of both *TNFRSF4* and *TNFSF4*, susceptibility for hypertension may be affected only by *TNFRSF4*, though more extensive studies are required before we conclude an association of *TNFSF4* with hypertension.

In the present study, we found that variations of *TNFRSF4* affected hypertension susceptibility only in women. This is an interesting similarity to women-specific MI susceptibility exerted by *TNFSF4* and *TNFRSF4*. Some case–control studies [5,60,61] have identified gene variants associated with sex-specific susceptibility to EH. Recently, Nakayama *et al.* [5] reported that an SNP in the 5′-untranslated region of the follicle-stimulating hormone receptor (*FSHR*) gene, in which mutations were reported to cause hereditary hypergonadotropic ovarian failure [62], was associated with EH in women and affected the levels of transcriptional activity. In this study, the functional mutation of the gene was clearly identified in patients with EH in a sex-specific manner. Currently, the reason for women-specific association of *TNFRSF4* with EH is an open question. One possibility is the involvement of the female sex hormone, estrogen. After menopause, women are at increased risk of inflammatory cardiovascular diseases such as atherosclerosis and coronary heart disease, suggesting that estrogens modulate the initiation and progress of inflammation [63–65]. Recently, Xing *et al.* [66] suggested that estrogen may exert anti-inflammatory effects by inhibiting tumor necrosis factor-α-mediated chemokine production in vascular smooth muscle cells. Estrogen, however, is also known to increase CRP, which is an inflammatory marker [63]. These findings indicate that estrogen may modulate the production of several proinflammatory molecules in distinct pathways. It is possible that TNFRSF4 and estrogen cross talk in inflammation networks.

In conclusion, the present study revealed that haplotypes of the *TNFRSF4* gene were associated with EH among women in two Japanese population, suggesting an involvement of the *TNFRSF4* gene in the pathogenesis of female EH.

Acknowledgements

We thank Naoto Ishii and Yoshihiro Onouchi for advice and assistance. We also thank Mieko Yoshida and Yoshiko Hotta for excellent technical assistance. This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

There are no conflicts of interest.

References

1 Kearney PM, Whelton M, Reynolds K, Muntner P, Whelton PK, He J. Global burden of hypertension: analysis of worldwide data. *Lancet* 2005; 365:217–223.
2 Tanira MO, Al Balushi KA. Genetic variations related to hypertension: a review. *J Hum Hypertens* 2005; 19:7–19.
3 Cowley AW Jr. The genetic dissection of essential hypertension. *Nat Rev Genet* 2006; 7:829–840.
4 Hubner N, Yagil C, Yagil Y. Novel integrative approaches to the identification of candidate genes in hypertension. *Hypertension* 2006; 47:1–5.
5 Nakayama T, Kuroi N, Sano M, Tabara Y, Katsuya T, Ogihara T, *et al.* Mutation of the follicle-stimulating hormone receptor gene 5′-untranslated region associated with female hypertension. *Hypertension* 2006; 48:512–518.
6 Calderhead DM, Buhlmann JE, van den Eertwegh AJ, Claassen E, Noelle RJ, Fell HP. Cloning of mouse Ox40: a T cell activation marker that may mediate T-B cell interactions. *J Immunol* 1993; 151:5261–5271.
7 Godfrey WR, Fagnoni FF, Harara MA, Buck D, Engleman EG. Identification of a human OX-40 ligand, a costimulator of CD4+ T cells with homology to tumor necrosis factor. *J Exp Med* 1994; 180:757–762.
8 Mallett S, Fossum S, Barclay AN. Characterization of the MRC OX40 antigen of activated CD4 positive T lymphocytes: a molecule related to nerve growth factor receptor. *EMBO J* 1990; 9:1063–1068.
9 Paterson DJ, Jefferies WA, Green JR, Brandon MR, Corthesy P, Puklavec M, *et al.* Antigens of activated rat T lymphocytes including a molecule of 50,000 Mr detected only on CD4 positive T blasts. *Mol Immunol* 1987; 24:1281–1290.
10 Miura S, Ohtani K, Numata N, Niki M, Ohbo K, Ina Y, *et al.* Molecular cloning and characterization of a novel glycoprotein, gp34, that is specifically induced by the human T-cell leukemia virus type I transactivator p40tax. *Mol Cell Biol* 1991; 11:1313–1325.
11 Akiba H, Miyahira Y, Atsuta M, Takeda K, Nohara C, Futagawa T, *et al.* Critical contribution of OX40 ligand to T helper cell type 2 differentiation in experimental leishmaniasis. *J Exp Med* 2000; 191:375–380.
12 Imura A, Hori T, Imada K, Ishikawa T, Tanaka Y, Maeda M, *et al.* The human OX40/gp34 system directly mediates adhesion of activated T cells to vascular endothelial cells. *J Exp Med* 1996; 183:2185–2195.
13 Stuber E, Neurath M, Calderhead D, Fell HP, Strober W. Cross-linking of OX40 ligand, a member of the TNF/NGF cytokine family, induces proliferation and differentiation in murine splenic B cells. *Immunity* 1995; 2:507–521.
14 Weinberg AD, Wegmann KW, Funatake C, Whitham RH. Blocking OX-40/OX-40 ligand interaction in vitro and in vivo leads to decreased T cell function and amelioration of experimental allergic encephalomyelitis. *J Immunol* 1999; 162:1818–1826.
15 Sugamura K, Ishii N, Weinberg AD. Therapeutic targeting of the effector T-cell co-stimulatory molecule OX40. *Nat Rev Immunol* 2004; 4:420–431.
16 Weinberg AD. OX40: targeted immunotherapy: implications for tempering autoimmunity and enhancing vaccines. *Trends Immunol* 2002; 23:102–109.
17 Weinberg AD, Evans DE, Thalhofer C, Shi T, Prell RA. The generation of T cell memory: a review describing the molecular and cellular events following OX40 (CD134) engagement. *J Leukoc Biol* 2004; 75:962–972.
18 Croft M. Co-stimulatory members of the TNFR family: keys to effective T-cell immunity? *Nat Rev Immunol* 2003; 3:609–620.
19 de Boer OJ, Becker AE, van der Wal AC. T lymphocytes in atherogenesis-functional aspects and antigenic repertoire. *Cardiovasc Res* 2003; 60:78–86.
20 Hansson GK, Libby P, Schonbeck U, Yan ZQ. Innate and adaptive immunity in the pathogenesis of atherosclerosis. *Circ Res* 2002; 91:281–291.
21 Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. *Circulation* 2002; 105:1135–1143.
22 Wang X, Ria M, Kelmenson PM, Eriksson P, Higgins DC, Samnegard A, *et al.* Positional identification of TNFSF4, encoding OX40 ligand, as a gene that influences atherosclerosis susceptibility. *Nat Genet* 2005; 37:365–372.
23 Ria M, Eriksson P, Boquist S, Ericsson CG, Hamsten A, Lagercrantz J. Human genetic evidence that OX40 is implicated in myocardial infarction. *Biochem Biophys Res Commun* 2006; 339:1001–1006.

24 Wilson PW. An epidemiologic perspective of systemic hypertension, ischemic heart disease, and heart failure. *Am J Cardiol* 1997; **80**: 3J–6J.
25 Samnegard A, Silveira A, Lundman P, Boquist S, Odeberg J, Hulthe J, *et al.* Serum matrix metalloproteinase-3 concentration is influenced by MMP-3-1612 5A/6A promoter genotype and associated with myocardial infarction. *J Intern Med* 2005; **258**:411–419.
26 Sugimoto K, Katsuya T, Ohkubo T, Hozawa A, Yamamoto K, Matsuo A, *et al.* Association between angiotensin II type 1 receptor gene polymorphism and essential hypertension: the Ohasama Study. *Hypertens Res* 2004; **27**:551–556.
27 1999 World Health Organization, International Society of Hypertension Guidelines for the Management of Hypertension. Guidelines Subcommittee. J Hypertens 1999; 17:151–183.
28 Kim HS, Krege JH, Kluckman KD, Hagaman JR, Hodgin JB, Best CF, *et al.* Genetic control of blood pressure and the angiotensinogen locus. *Proc Natl Acad Sci U S A* 1995; **92**:2735–2739.
29 Kim HS, Lee G, John SW, Maeda N, Smithies O. Molecular phenotyping for analyzing subtle genetic effects in mice: application to an angiotensinogen gene titration. *Proc Natl Acad Sci U S A* 2002; **99**:4602–4607.
30 Bannai M, Higuchi K, Akesaka T, Furukawa M, Yamaoka M, Sato K, *et al.* Single-nucleotide-polymorphism genotyping for whole-genome-amplified samples using automated fluorescence correlation spectroscopy. *Anal Biochem* 2004; **327**:215–221.
31 Fujii K, Matsubara Y, Akanuma J, Takahashi K, Kure S, Suzuki Y, *et al.* Mutation detection by TaqMan-allele specific amplification: application to molecular diagnosis of glycogen storage disease type Ia and medium-chain acyl-CoA dehydrogenase deficiency. *Hum Mutat* 2000; **15**:189–196.
32 Barbieri M, Ferrucci L, Corsi AM, Macchi C, Lauretani F, Bonafe M, *et al.* Is chronic inflammation a determinant of blood pressure in the elderly? *Am J Hypertens* 2003; **16**:537–543.
33 Derhaschnig U, Shehata M, Herkner H, Bur A, Woisetschlager C, Laggner AN, *et al.* Increased levels of transforming growth factor-beta1 in essential hypertension. *Am J Hypertens* 2002; **15**:207–211.
34 Ferri C, Desideri G, Baldoncini R, Bellini C, De Angelis C, Mazzocchi C, *et al.* Early activation of vascular endothelium in nonobese, nondiabetic essential hypertensive patients with multiple metabolic abnormalities. *Diabetes* 1998; **47**:660–667.
35 Festa A, D'Agostino R Jr, Howard G, Mykkanen L, Tracy RP, Haffner SM. Chronic subclinical inflammation as part of the insulin resistance syndrome: the Insulin Resistance Atherosclerosis Study (IRAS). *Circulation* 2000; **102**:42–47.
36 Lakoski SG, Cushman M, Palmas W, Blumenthal R, D'Agostino RB Jr, Herrington DM. The relationship between blood pressure and C-reactive protein in the Multi-Ethnic Study of Atherosclerosis (MESA). *J Am Coll Cardiol* 2005; **46**:1869–1874.
37 Parissis JT, Korovesis S, Giazitzoglou E, Kalivas P, Katritsis D. Plasma profiles of peripheral monocyte-related inflammatory markers in patients with arterial hypertension. Correlations with plasma endothelin-1. *Int J Cardiol* 2002; **83**:13–21.
38 Pockley AG, De Faire U, Kiessling R, Lemne C, Thulin T, Frostegard J. Circulating heat shock protein and heat shock protein antibody levels in established hypertension. *J Hypertens* 2002; **20**:1815–1820.
39 Ridker PM, Buring JE, Cook NR, Rifai N. C-reactive protein, the metabolic syndrome, and risk of incident cardiovascular events: an 8-year follow-up of 14 719 initially healthy American women. *Circulation* 2003; **107**:391–397.
40 Sung KC, Suh JY, Kim BS, Kang JH, Kim H, Lee MH, *et al.* High sensitivity C-reactive protein as an independent risk factor for essential hypertension. *Am J Hypertens* 2003; **16**:429–433.
41 Yan JC, Ma GS, Wu ZG, Kong XT, Zong RQ, Zhan LZ. Increased levels of CD40–CD40 ligand system in patients with essential hypertension. *Clin Chim Acta* 2005; **355**:191–196.
42 Dorffel Y, Latsch C, Stuhlmuller B, Schreiber S, Scholze S, Burmester GR, *et al.* Preactivated peripheral blood monocytes in patients with essential hypertension. *Hypertension* 1999; **34**:113–117.
43 Grundy SM. Inflammation, hypertension, and the metabolic syndrome. *JAMA* 2003; **290**:3000–3002.
44 Li JJ. Inflammation in hypertension: primary evidence. *Chin Med J (Engl)* 2006; **119**:1215–1221.
45 Li JJ, Fang CH, Hui RT. Is hypertension an inflammatory disease? *Med Hypotheses* 2005; **64**:236–240.
46 Sesso HD, Buring JE, Rifai N, Blake GJ, Gaziano JM, Ridker PM. C-reactive protein and the risk of developing hypertension. *JAMA* 2003; **290**:2945–2951.
47 Bataillard A, Renaudin C, Sassard J. Silica attenuates hypertension in Lyon hypertensive rats. *J Hypertens* 1995; **13**:1581–1584.
48 Bush E, Maeda N, Kuziel WA, Dawson TC, Wilcox JN, DeLeon H, *et al.* CC chemokine receptor 2 is required for macrophage infiltration and vascular hypertrophy in angiotensin II-induced hypertension. *Hypertension* 2000; **36**:360–363.
49 Clozel M, Kuhn H, Hefti F, Baumgartner HR. Endothelial dysfunction and subendothelial monocyte macrophages in hypertension. Effect of angiotensin converting enzyme inhibition. *Hypertension* 1991; **18**:132–141.
50 Haller H, Behrend M, Park JK, Schaberg T, Luft FC, Distler A. Monocyte infiltration and c-fms expression in hearts of spontaneously hypertensive rats. *Hypertension* 1995; **25**:132–138.
51 Hilgers KF, Hartner A, Porst M, Mai M, Wittmann M, Hugo C, *et al.* Monocyte chemoattractant protein-1 and macrophage infiltration in hypertensive kidney injury. *Kidney Int* 2000; **58**:2408–2419.
52 Johnson RJ, Alpers CE, Yoshimura A, Lombardi D, Pritzl P, Floege J, *et al.* Renal injury from angiotensin II-mediated hypertension. *Hypertension* 1992; **19**:464–474.
53 McCarron RM, Wang L, Siren AL, Spatz M, Hallenbeck JM. Monocyte adhesion to cerebromicrovascular endothelial cells derived from hypertensive and normotensive rats. *Am J Physiol* 1994; **267**:H2491–H2497.
54 Schmid-Schonbein GW, Seiffge D, DeLano FA, Shen K, Zweifach BW. Leukocyte counts and activation in spontaneously hypertensive and normotensive rats. *Hypertension* 1991; **17**:323–330.
55 Herrington DM, Brown WV, Mosca L, Davis W, Eggleston B, Hundley WG, *et al.* Relationship between arterial stiffness and subclinical aortic atherosclerosis. *Circulation* 2004; **110**:432–437.
56 van Popele NM, Grobbee DE, Bots ML, Asmar R, Topouchian J, Reneman RS, *et al.* Association between arterial stiffness and atherosclerosis: the Rotterdam Study. *Stroke* 2001; **32**:454–460.
57 Dernellis J, Panaretou M. Aortic stiffness is an independent predictor of progression to hypertension in nonhypertensive subjects. *Hypertension* 2005; **45**:426–431.
58 Liao D, Arnett DK, Tyroler HA, Riley WA, Chambless LE, Szklo M, *et al.* Arterial stiffness and the development of hypertension. The ARIC study. *Hypertension* 1999; **34**:201–206.
59 Franklin SS. Arterial stiffness and hypertension: a two-way street? *Hypertension* 2005; **45**:349–351.
60 O'Donnell CJ, Lindpaintner K, Larson MG, Rao VS, Ordovas JM, Schaefer EJ, *et al.* Evidence for association and genetic linkage of the angiotensin-converting enzyme locus with hypertension and blood pressure in men but not women in the Framingham Heart Study. *Circulation* 1998; **97**:1766–1772.
61 Ono K, Mannami T, Iwai N. Association of a promoter variant of the haeme oxygenase-1 gene with hypertension in women. *J Hypertens* 2003; **21**:1497–1503.
62 Aittomaki K, Lucena JL, Pakarinen P, Sistonen P, Tapanainen J, Gromoll J, *et al.* Mutation in the follicle-stimulating hormone receptor gene causes hereditary hypergonadotropic ovarian failure. *Cell* 1995; **82**:959–968.
63 Stork S, van der Schouw YT, Grobbee DE, Bots ML. Estrogen, inflammation and cardiovascular risk in women: a critical appraisal. *Trends Endocrinol Metab* 2004; **15**:66–72.
64 Rosano GM, Vitale C, Marazzi G, Volterrani M. Menopause and cardiovascular disease: the evidence. *Climacteric* 2007; **10 (Suppl 1)**:19–24.
65 Nilsson BO. Modulation of the inflammatory response by estrogens with focus on the endothelium and its interactions with leukocytes. *Inflamm Res* 2007; **56**:269–273.
66 Xing D, Feng W, Miller AP, Weathington NM, Chen YF, Novak L, *et al.* Estrogen modulates TNF-alpha-induced inflammatory responses in rat aortic smooth muscle cells through estrogen receptor-beta activation. *Am J Physiol Heart Circ Physiol* 2007; **292**:H2607–H2612.

Original Article

Prevalence and Lifestyle Characteristics of Hypertensive Patients with Metabolic Syndrome Followed at an Outpatient Clinic in Fukuoka, Japan

Yuko OHTA[1)], Takuya TSUCHIHASHI[1)], Kimika ARAKAWA[1)], Uran ONAKA[1)], and Michio UENO[1)]

A new guideline on metabolic syndrome (MS) in Japanese was introduced in 2005. The purpose of this study was to evaluate the prevalence and lifestyle characteristics of Japanese hypertensive patients with MS. Subjects were 290 patients (mean age: 64±11 years) who had been followed at our hospital. The waist circumference (WC) and body mass index (BMI) were assessed. Subjects who had BMI ≥25 kg/m² were defined as having BMI obesity, while abdominal obesity was defined as a WC ≥85 cm in men and ≥90 cm in women, respectively. Since all patients had hypertension, the definition of MS was made when the patient had abdominal obesity plus either dyslipidemia or glucose intolerance, or both. Among the subjects, 230 patients underwent 24-h home urine collection to measure urinary salt and potassium excretions. Dietary habits were also assessed by use of a questionnaire. Mean values of BMI and WC were 24.2±3.4 kg/m² and 87.1±9.6 cm, respectively. Among the total subject group, 39% patients were classified as having BMI obesity, 49% as having abdominal obesity, and 27% as having MS. BMI was significantly correlated with WC both in men (r=0.86; p<0.01) and in women (r=0.79; p<0.01). More men than women belonged to the BMI obesity (46% *vs.* 33%, p<0.05), abdominal obesity (63% *vs.* 39%, p<0.01) and MS (39% *vs.* 18%, p<0.01) groups. There were no significant differences in blood pressure between patients with and without MS, while patients with MS needed a greater number of antihypertensive drugs than those without MS. Mean urinary salt and potassium excretions were 8.9±3.8 g/day and 1.9±0.7 g/day, respectively. Urinary salt excretion of <6 g (100 mmol of sodium)/day was achieved in 20% of the subjects. Urinary salt excretion in the patients with MS was significantly higher than that in the patients without (10.1±4.2 *vs.* 8.5±3.6 g/day; p<0.01). Only 16% of the patients with MS achieved salt restriction (<6 g/day). The patients with MS had a significantly greater the chance to eat out than the patients without MS. They were also less aware of the need to increase their vegetable consumption. The results suggested that MS is prevalent in Japanese hypertensive patients. Patients with MS showed higher urinary salt excretion and needed more antihypertensive drugs to manage their blood pressure. Dietary counseling focusing not only on sodium restriction but also on the need to increase fruit and vegetable consumption seems to be important. (*Hypertens Res* 2007; 30: 1077–1082)

Key Words: **metabolic syndrome, hypertensive patients, abdominal obesity, urinary salt excretion**

From the [1)]Division of Hypertension, Clinical Research Center, National Kyushu Medical Center, Fukuoka, Japan.
Address for Reprints: Yuko Ohta, M.D., Division of Hypertension, Clinical Research Center, National Kyushu Medical Center, Jigyohama 1–8–1, Chuo-ku, Fukuoka 810–8563, Japan. E-mail: yukoo@qmed.hosp.go.jp
Received December 28, 2006; Accepted in revised form June 29, 2007.

Introduction

In recent years, the prevalence of glucose intolerance, hypercholesterolemia, and obesity has greatly increased in Japan (*1*). The concept and the definition of metabolic syndrome (MS) have been established by the National Cholesterol Education Program's Adult Treatment Panel III (*2*) and by WHO (*3*). Following these guidelines, a guideline on MS in Japanese was introduced in 2005 (*4*). MS has been reported to be associated with an increased risk for type 2 diabetes mellitus, cardiovascular disease and other manifestations of atherosclerotic disease (*5–10*). Since lifestyle factors can make a major contribution to the development of MS, dietary modification and enhanced physical activity may delay or prevent the disease (*2, 11–13*).

Among the relevant lifestyle factors, dietary sodium intake plays a major role in the development of hypertension. Although the National Nutrition Survey in Japan showed a tendency for decreased sodium intake in recent years, it still remains high (*14*). In the present study, we evaluated the prevalence and lifestyle characteristics—including sodium intake—of Japanese hypertensive patients with MS.

Methods

Participants were recruited from among hypertensive outpatients who visited the National Kyushu Medical Center, Fukuoka, Japan. Subjects included 290 patients, 167 women and 123 men, with a mean age of 64±11 years. The waist circumference (WC) and body mass index (BMI) were assessed. WC was measured at the umbilical level in standing subjects after normal expiration. Subjects who had a BMI ≥25 kg/m^2 were defined as having BMI obesity, while abdominal obesity was defined as a WC ≥85 cm in men and ≥90 cm in women, respectively. Blood pressure (BP) was measured with a sphygmomanometer by the doctors while the patients were seated. The averaged BP determined by two consecutive measurements was used for analysis. Body fat was measured by bioelectric impedance analysis (InBody 3.0; Biospace Tokyo Japan Inc., Tokyo, Japan). Since all patients had hypertension (systolic blood pressure [SBP] ≥130 mmHg and/or diastolic blood pressure [DBP] ≥85 mmHg or current use of antihypertensive drugs), the definition of MS was made when the patient had abdominal obesity plus either dyslipidemia (serum triglyceride ≥150 mg/dL and/or serum high-density lipoprotein [HDL] cholesterol <40 mg/dL or the current use of anti-lipemic agents) or glucose intolerance (fasting plasma glucose ≥110 mg/dL or the current use of anti-diabetic agents), or both.

In the second part of the analysis, 24-h urine samples were collected using a partition cup, which collects a 1/50 portion of the 24-h urine. If the 24-h creatinine excretion was within ±30% of the estimated values, the urine collection was considered successful. Subjects included 230 patients who underwent successful 24-h home urine collection. Urinary salt, potassium, and creatinine were measured. Dietary habits focusing on the chance to eat out (≥1/week) and the awareness of the need to increase vegetable and fruit consumption were also assessed by use of a questionnaire.

The detailed procedure of the study was explained and informed consent was obtained from each subject. This study was conducted following the institutional guidelines.

Statistical Analysis

Values are presented as the mean±SD. The differences in the variables were compared by one-way ANOVA or generalized linear model (GLM) when applicable. A χ^2 test was also utilized when appropriate. p values less than 0.05 were considered significant.

Results

The patient characteristics are shown in Table 1. The mean age was 64±11 years, and 58% of the patients were women. The mean values of BMI and WC were 24.2±3.4 kg/m^2 and 87.1±9.6 cm, respectively. Among the total patient group, 39% were classified as having BMI obesity, 49% as having abdominal obesity, and 27% as having MS. More men than women belonged to the BMI obesity (46% *vs.* 33%, $p<0.05$), abdominal obesity (63% *vs.* 39%, $p<0.01$) and MS (39% *vs.* 18%, $p<0.01$) groups. BMI was significantly correlated with WC both in men ($r=0.86$; $p<0.01$) and in women ($r=0.79$; $p<0.01$). Table 2 compares the profiles of hypertensive patients with and without MS. As a matter of course, WC, serum triglyceride and plasma glucose were significantly higher and HDL cholesterol was significantly lower in both male and female patients with MS than those without MS. The values of total cholesterol and uric acid in men and BMI in women were significantly higher in the MS group. On the other hand, there were no significant differences in BP between patients with and without MS, while patients with MS needed a greater number of antihypertensive drugs than those without MS (men: 2.0±1.3 *vs.* 1.6±1.0, $p=0.10$; women: 2.1±1.0 *vs.* 1.6±1.1, $p<0.05$). As shown in Fig. 1, angiotensin II receptor blockers and α-blockers were prescribed more frequently in the patients with MS. Table 3 shows the characteristics of the patients with and without MS who underwent successful 24-h home urine collection. The mean urinary salt and potassium excretions of all patients who underwent successful 24-h home urine collection were 8.9±3.8 g/day and 1.9±0.7 g/day, respectively. A urinary salt excretion of <6 g (100 mmol of sodium)/day was achieved in 20% of the subjects. The urinary salt excretion in the patients with MS was significantly higher than that in the patients without MS (10.1±4.2 *vs.* 8.5±3.6 g/day; $p<0.01$). Only 16% of the patients with MS achieved salt restriction (<6 g/day). The patients with MS had a significantly greater chance to eat out than the patients without MS (Table 3). They were

Table 1. Characteristics of the Patients

	Total	Men	Women
Number of patients	290	123	167
Age (years)	64±11	63±12	65±10
BMI (kg/m²)	24.2±3.4	24.4±3.3	24.0±3.5
Waist circumference (cm)	87.1±9.6	87.9±8.6	86.5±10.2
Serum total cholesterol (mg/dL)	205±30	199±31	210±28**
Serum triglyceride (mg/dL)	135±89	145±84	127±93
Serum HDL cholesterol (mg/dL)	59±16	54±14	62±17**
Serum creatinine (mg/dL)	0.8±0.4	1.0±0.4	0.7±0.3**
Serum uric acid (mg/dL)	5.9±1.4	6.7±1.3	5.3±1.1**
Plasma glucose (mg/dL)	102±19	103±16	101±21
Systolic blood pressure (mmHg)	135±16	134±13	136±17
Diastolic blood pressure (mmHg)	71±11	71±10	71±11
Number of antihypertensive drugs	1.7±1.1	1.8±1.1	1.7±1.1
Prevalence of BMI obesity (%)	39	46	33*
Prevalence of AO (%)	49	63	39**
Prevalence of MS (%)	27	39	18**

Values are means±SD. *$p<0.05$, **$p<0.01$ *vs.* men. BMI, body mass index; HDL, high-density lipoprotein; AO, abdominal obesity; MS, metabolic syndrome.

Table 2. Characteristics of the Patients with and without Metabolic Syndrome

	Men		Women	
	Non-MS	MS	Non-MS	MS
Number of patients	75	48	137	30
Age (years)	65±11	61±12**	64±10	67±8
Body mass index (kg/m²)	23.1±3.1	26.6±2.3	23.3±3.2	27.1±3.2**
Waist circumference (cm)	84.2±8.2	93.6±5.6††	83.8±9.0	98.4±6.4††
Serum total cholesterol (mg/dL)	194±31	207±30†	212±27	201±31
Serum triglyceride (mg/dL)	106±58	205±84††	116±67	177±156††
Serum HDL cholesterol (mg/dL)	58±14	48±13††	63±17	55±15†
Serum creatinine (mg/dL)	1.0±0.4	1.0±0.3	0.7±0.3	0.7±0.3
Serum uric acid (mg/dL)	6.5±1.3	7.1±1.3†	5.3±1.1	5.3±1.0†
Plasma glucose (mg/dL)	99±12	110±19††	96±14	120±34††
Systolic blood pressure (mmHg)	135±12	132±15	135±17	138±16
Diastolic blood pressure (mmHg)	71±11	72±9	71±11	70±11
Number of antihypertensive drugs	1.6±1.0	2.0±1.3	1.6±1.1	2.1±1.0†

Values are means±SD. *$p<0.05$, **$p<0.01$ *vs.* non-MS. †$p<0.05$, ††$p<0.01$ *vs.* non-MS adjusted for age and body mass index. MS, metabolic syndrome.

also less aware of the need to increase their vegetable consumption (Table 3). Similarly, the patients with MS tended to be less aware of the need to increase fruit consumption (Table 3).

Discussion

In our present group of hypertensive patients, we found that the prevalence of MS was 39% for men and 18% for women. Epidemiological studies have shown that MS occurs in a wide variety of ethnic groups (*12, 15–18*). Data extracted from the third National Health and Nutrition Examination Survey (*12*) have shown that the age-adjusted prevalence of MS is 24% as defined by the NCEP-ATP III definition of MS. The prevalence of MS in the Hungarian population (*16*) and in Okayama Prefecture (*18*) have been reported as 15% and 31% for men, and 9% and 4% for women, respectively. The prevalence of MS in the present study was higher than any of these previously reported values. This finding was expected given that all our subjects were hypertensive. Our study used the new definition of MS for Japanese, which primarily requires a WC ≥85 cm in men and ≥90 cm in women in addition to

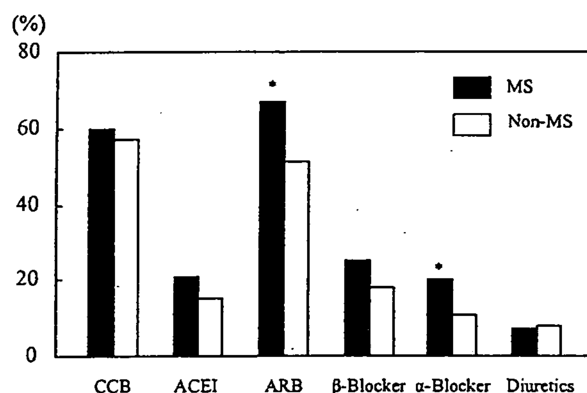


Fig. 1. *Antihypertensive drugs used in the patients with and without MS. *p < 0.05 vs. non-MS. MS, metabolic syndrome; CCB, Ca antagonist; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin II receptor blocker.*

Table 3. Characteristics of the Patients with and without MS Who Underwent 24-h Urine Collection

	Non-MS	MS
Number of patients	175	55
Sex (men/women)	66/109	37/18**
Age (years)	64±10	63±12
Body mass index (kg/m²)	23.3±2.9	26.8±2.5**
Body fat (%)	28±7	29±6
Waist circumference (cm)	84.4±8.3	95.0±6.3**
Systolic blood pressure (mmHg)	135±16	134±14
Diastolic blood pressure (mmHg)	71±11	71±10
Serum creatinine (mg/dL)	0.8±0.4	0.9±0.4
Urinary salt excretion (g/day)	8.5±3.6	10.1±4.2**
Urinary potassium excretion (g/day)	1.9±0.7	1.9±0.6
Urinary creatinine excretion (mg/day)	944±274	1,187±360
Chance to eat out (≥1/week) (%)		
All	41.9	72.3††
Men	52.7	78.1†
Women	35.5	60.0#
Awareness to increase vegetable consumption (%)		
All	75.4	60.0†
Men	71.2	62.2
Women	78.0	55.6†
Awareness to increase fruit consumption (%)		
All	73.1	52.7#
Men	59.1	43.2
Women	81.7	72.2

Values are means±SD. MS, metabolic syndrome. $^{**}p<0.01$ *vs.* non-MS. $^{\#}p<0.1$, $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ *vs.* non-MS (adjusted for age).

two or more of the following risk factors: hypertension, dyslipidemia, and glucose intolerance. These cutoff points of the WC are reported to correspond to a visceral fat area of 100 cm² at the umbilical level (*19*). Although the prevalence of MS varies by the definition used and the population studied, there is strong epidemiological evidence suggesting that the number of subjects with MS is increasing rapidly in many countries.

In the present study, the prevalence of abdominal obesity differed from that of BMI obesity; however, there was a significant association between abdominal obesity and BMI obesity. Based on the regression analysis, BMI of 25 kg/m² corresponded to a WC of 89.3 cm in men and 88.7 cm in women, respectively. These WC values were close to the criteria for abdominal obesity in the new guidelines on MS in Japanese.

Since multiple risk factors for cardiovascular disease exist in patients with MS, aggressive pharmacological as well as non-pharmacological intervention to achieve strict BP control should be required. In the present study, BP levels were comparable between the patients with and without MS, while the patients with MS required a greater number of antihypertensive drugs. Our results are consistent with previous studies in which obese patients required a greater number of antihypertensive drugs (*20, 21*). Among the lifestyle modifications recommended for hypertensive patients, sodium restriction seems to be the most important. One of the main findings of this study was that urinary salt excretion in the patients with MS was significantly higher than that in the patients without MS. The rate of achievement of urinary salt excretion <6 g/day in the patients with MS was also low. We have previously reported that sodium intake in Japanese hypertensive patients remains fairly high, and that very few of our patients were able to achieve the sodium restriction recommended by the guidelines (*22, 23*). Recently, it has been reported that the prevalence of sodium-sensitive hypertension is significantly higher in patients with MS than in those without MS, and the patients with MS show higher homeostasis model assessment of insulin resistance (HOMA-IR) (*24*). These findings indicate the close association between sodium-sensitive hypertension and insulin resistance. Taken together, these results indicate that sodium restriction in the hypertensive patients with MS may have an additional therapeutic advantage to reduce the risk of cardiovascular complication. In addition, the present patients with MS had a significantly greater chance to eat out, and were also less aware of the need to increase vegetable and fruit consumption. However, the urinary potassium excretion in the patients with MS was equal to that in the patients without MS in the present study, in spite of the reduced awareness of the need to increase vegetable consumption in the former group. One possible explanation for this finding is that actual potassium intake tends to differ from the natural content of food because of the influence of cooking. Some previous studies have reported on the association between food intake and MS (*11, 25–28*). A whole array of dietary factors, such as high intakes of saturated fatty acids and low intakes of n-3 fatty acids, have been reported to con-

tribute to the development of components of MS (*26, 27*). It has also been reported that the Dietary Approaches to Stop Hypertension (DASH) diet and the Mediterranean-style diet might be effective in reducing the prevalence of MS (*11, 28*). In addition, Riccardi and Rivellese introduced a diet with high amounts of vegetables, fruits, and legumes as well as reductions in saturated fat, salt, and alcohol as an optimal diet for MS (*29*). Thus, lifestyle modifications to target not only weight reduction, dietary sodium reduction, regular aerobic physical activity, and decreased alcohol consumption, but also increased fruit and vegetable consumption, may be helpful in hypertensive patients with MS.

One of the limitations of this study is that all subjects were hypertensives, and thus we did not examine the prevalence and lifestyle characteristics of normotensive patients with MS. Another limitation is that the present investigation was conducted in a hospital in Fukuoka. Therefore, our observations may not reflect the prevalence and lifestyle characteristics of hypertensive patients with MS in the general population.

In conclusion, our findings suggest that MS is prevalent in Japanese hypertensive patients. We found that our patients with MS required more antihypertensive drugs to manage BP than our hypertensive patients without MS. Urinary salt excretion was also high in the hypertensive patients with MS. These results indicate that dietary counseling focusing not only on sodium restriction but also on the value of increasing fruit and vegetable consumption is important.

References

1. Kubo M, Kiyohara Y, Kato I, *et al*: Trends in the incidence, mortality, and survival rate of cardiovascular disease in a Japanese community: Hisayama study. *Stroke* 2003; **34**: 2349–2354.
2. National Cholesterol Education Program (NCEP) Expert Panel: Third report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. *Circulation* 2002; **106**: 3143–3421.
3. Alberti KG, Zimmet PZ, for the WHO Consultation: Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med* 1998; **15**: 539–553.
4. Committee to Evaluate Diagnostic Standards for Metabolic Syndrome: Definition and the diagnostic standard for metabolic syndrome. *Nippon Naika Gakkai Zassi* 2005; **94**: 794–809 (in Japanese).
5. Solymoss BC, Bourassa MG, Campeau L, *et al*: Effect of increasing metabolic syndrome score on atherosclerotic risk profile and coronary artery disease angiographic severity. *Am J Cardiol* 2004; **93**: 159–164.
6. Lakka HM, Laaksonen DE, Lakka TA, *et al*: The metabolic syndrome and total and cardiovascular disease mortality in middle-aged men. *JAMA* 2002; **288**: 2709–2716.
7. Laaksonen DE, Lakka HM, Niskanen LK, Kaplan GA, Salonen JT, Lakka TA: Metabolic syndrome and development of diabetes mellitus: application and validation of recently suggested definitions of the metabolic syndrome in a prospective cohort study. *Am J Epidemiol* 2002; **156**: 1070–1077.
8. Isomaa B, Almgren P, Tuomi T, *et al*: Cardiovascular morbidity and mortality associated with the metabolic syndrome. *Diabetes Care* 2001; **24**: 683–689.
9. Malik S, Wong ND, Franklin SS, *et al*: Impact of the metabolic syndrome on mortality from coronary heart disease, cardiovascular disease, and all causes in United States adults. *Circulation* 2004; **110**: 1245–1250.
10. Takeuchi H, Saitoh S, Takagi S, *et al*: Metabolic syndrome and cardiac disease in Japanese men: applicability of the concept of metabolic syndrome defined by the National Cholesterol Education Program–Adult Treatment Panel III to Japanese Men—the Tanno and Sobetsu Study. *Hypertens Res* 2005; **28**: 203–208.
11. Azadbakht L, Mirmiran P, Esmaillzadeh A, Azizi T, Azizi F: Beneficial effect of a dietary approaches to stop hypertension eating plan on features of the metabolic syndrome. *Diabetes Care* 2005; **28**: 2823–2831.
12. Ford ES, Giles WH, Dietz WH: Prevalence of the metabolic syndrome among US adults: findings from the third national health and nutrition examination survey. *JAMA* 2002; **287**: 356–359.
13. Grundy SM, Cleeman JI, Daniels SR, *et al*: Diagnosis and management of the metabolic syndrome. An American Heart Association/National Heart, Lung, and Blood Institute scientific statement. Executive summary. *Circulation* 2005; **112**: 2735–2740.
14. Ministry of Health, Labour and Welfare, Japan: The National Nutrition Survey in Japan, 2002. Tokyo, Dai-ichi Shuppan Press, 2004, p 182.
15. Nakanishi N, Shiraishi T, Wada M: Brachial-ankle pulse wave velocity and metabolic syndrome in a Japanese population: the Minoh study. *Hypertens Res* 2005; **28**: 125–131.
16. Császár A, Kékes E, Abel T, Papp R, Kiss I, Balogh S: Prevalence of metabolic syndrome estimated by international diabetes federation criteria in a Hungarian population. *Blood Press* 2006; **15**: 101–106.
17. Aizawa Y, Kamimura N, Watanabe H, *et al*: Cardiovascular risk factors are really linked in the metabolic syndrome: this phenomenon suggests clustering rather than coincidence. *Int J Cardiol* 2006; **109**: 213–218.
18. Miyatake N, Kawasaki Y, Nishikawa H, Takenami S, Numata T: Prevalence of metabolic syndrome in Okayama prefecture, Japan. *Intern Med* 2006; **45**: 107–108.
19. The Examination Committee of Criteria for 'Obesity Disease' in Japan, Society for the Study of Obesity: New criteria for 'obesity disease' in Japan. *Circ J* 2002; **66**: 987–992.
20. Saito I, Murata K, Hirose H, Tsujioka M, Kawabe H: Relation between blood pressure control, body mass index, and intensity of medical treatment. *Hypertens Res* 2003; **26**: 711–715.
21. Modan M, Almog S, Fuchs Z, Chetrit A, Lusky A, Halkin H: Obesity, glucose intolerance, hyperinsulinemia, and response to antihypertensive drugs. *Hypertension* 1991; **17**: 565–573.

22. Ohta Y, Tsuchihashi T, Ueno M, *et al*: Relationship between the awareness of salt restriction and the actual salt intake in hypertensive patients. *Hypertens Res* 2004; **27**: 243–246.
23. Ohta Y, Tsuchihashi T, Onaka U, Eto K, Tominaga M, Ueno M: Long-term compliance of salt restriction in Japanese hypertensive patients. *Hypertens Res* 2005; **28**: 953–957.
24. Uzu T, Kimura G, Yamauchi A, *et al*: Enhanced sodium sensitivity and disturbed circadian rhythm of blood pressure in essential hypertension. *J Hypertens* 2006; **24**: 1627–1632.
25. Azadbakht L, Mirmiran P, Esmaillzadeh A, Azizi F: Dairy consumption is inversely associated with the prevalence of the metabolic syndrome in Tehranian adults. *Am J Clin Nutr* 2005; **82**: 523–530.
26. Vessby B: Dietary fat and insulin action in humans. *Br J Nutr* 2000; **83**: S91–S96.
27. Connor WE: Importance of n-3 fatty acids in health and disease. *Am J Clin Nutr* 2000; **71**: S171–S175.
28. Esposito K, Marfella R, Ciotola M, *et al*: Effect of a Mediterranean-style diet on endothelial dysfunction and markers of vascular inflammation in the metabolic syndrome: a randomized trial. *JAMA* 2004; **292**: 1440–1446.
29. Riccardi G, Rivellese AA: Dietary treatment of the metabolic syndrome—the optimal diet. *Br J Nutr* 2000; **83**: S143–S148.