ORIGINAL ARTICLE

Association of gene polymorphism of the fat-mass and obesity-associated gene with insulin resistance in Japanese

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It was reported that gene polymorphisms in the fat-mass and obesity-associated gene (FTO) were associated with obesity and diabetes in several genome-wide association studies. A recent report indicated that FTO-knockout mice exhibited phenotypes of skinny body shape and normal metabolic profiles. Thus, FTO could be important in metabolic disorders. The aim of this study was to clarify the role of single nucleotide polymorphisms (SNPs) in FTO in metabolic disorders such as hypertension, obesity, diabetes, dyslipidemia, insulin resistance and metabolic syndrome in the Japanese general population using data from a cohort study in Hokkaido, namely the Tanno–Sobetsu study. Written informed consent for the genetic analysis was obtained from each subject participating in the study. A total of 1514 subjects were genotyped by TaqMan PCR methods for three SNPs, rs9939609, rs1121980 and rs1558902, in FTO. Association analyses between the SNPs and metabolic parameters were performed. Although two SNPs, rs9939609 and rs1558902, were not significantly associated with hypertension, obesity, metabolic syndrome or any metabolic parameters, additive and recessive models of rs1121980 were strongly associated with plasma immunoreactive insulin (IRI) level and homeostasis model assessment insulin resistance (HOMA-IR), even after adjusting for confounding factors such as age, gender and body mass index. A haplotype of three SNPs was also significantly associated with IRI and HOMA-IR. One SNP, rs1121980, and a haplotype of three SNPs in FTO that contains this SNP, might be important in the progression of insulin resistance in Japanese subjects.
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INTRODUCTION

Metabolic syndrome (MS) consisting of central obesity, high blood pressure, abnormal glucose tolerance or abnormal lipid profiles is considered an independent risk factor for cardiovascular diseases such as ischemic heart disease and stroke.[1,2] In Japan, central obesity based on visceral fat accumulation is an essential diagnosis criterion for MS. One of the main pathways to central obesity is considered to be an imbalance in the secretion of adipocytokines from adipose tissues and subsequent following insulin resistance.[3] Genetic background influences metabolic disorders. Recently, several genome-wide association studies revealed that single nucleotide polymorphisms (SNPs) of the fat-mass and obesity-associated gene (FTO) might be predisposing factors for obesity, diabetes and MS.[4–6]

Recently, it was reported that FTO-knockout mice showed several characteristic phenotypes, which included skinny body shape and normal metabolic profiles.[7] Thus, FTO could be important in fat accumulation and the regulation of glucose or lipid metabolism.

In previous genetic analyses, SNPs rs9939609, rs1121980 and rs1558902 in FTO were strongly associated with obesity, defined by body mass index (BMI).[8–11] There remain questions concerning whether these SNPs affect obesity and other metabolic disorders in Japanese subjects, who have a quite different body shape and diet from subjects in Western countries. Furthermore, the allele frequencies of FTO SNPs in Japanese subjects are quite different from those in Caucasian subjects (http://hapmap.ncbi.nlm.nih.gov/).

It was reported by Japanese investigators that rs1558902, but not rs9939609, in FTO was associated with BMI in a case (severe obesity group: BMI $\geqslant$ 30 kg m^{-2})–control (nonobesity group: BMI $\leqslant$ 23 kg m^{-2}) study.[9] However, there are no reports on investigations of the relationship between FTO SNPs and metabolic disorders including hypertension, obesity, DM and MS as well as other parameters such as insulin resistance in the Japanese general population. In this study, we investigate the role of FTO SNPs in metabolic disorders in a cohort study, namely the Tanno–Sobetsu study in Hokkaido, northern Japan.

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METHODS

Study subjects

We recruited 1514 subjects (803 in Tanno town and 711 in Sobetsu town) who had undergone medical checkups in these towns in Hokkaido, Japan, in 2002. The detailed epidemiological findings have already been reported.[12-17] Subjects completed a standard questionnaire regarding their medical history, and smoking and drinking habits. We measured the systolic blood pressure (SBP), diastolic blood pressure (DBP), BMI, abdominal circumference, total cholesterol, triglyceride, high-density lipoprotein cholesterol, plasma glucose and immunoreactive insulin (IRI). Blood samples were collected during fasting in the early morning. Obesity was defined as a BMI > 25 kg m^{-2}. Dyslipidemia was defined as total cholesterol $\geqslant 220$ mg per 100 ml and/or drug treatment for hypercholesterolemia. Diabetes was defined as fasting blood sugar $\geqslant 126$ mg per 100 ml and/or drug treatment for hyperglycemia. The Japanese definition of MS[18] was used as the diagnosis for MS. Briefly, criterion 1 and two of criteria 2–4 needed to be met.

1. visceral fat: (male) abdominal circumference $\geqslant 85$ cm
   (female) abdominal circumference $\geqslant 90$ cm
2. lipid abnormality: treatment for dyslipidemia or
   triglyceride $\geqslant 150$ mg per 100 ml and/or high-density lipoprotein cholesterol < 40 mg per 100 ml
3. blood pressure: treatment for hypertension or
   SBP $\geqslant 130$ and/or DBP $\geqslant 85$ mm Hg
4. hyperglycemia: treatment for diabetes or fasting blood sugar $\geqslant 110$ mg per 100 ml.

Homeostasis model assessment insulin resistance (HOMA-IR) was used to determine insulin sensitivity, and was calculated as plasma glucose (mg per 100 ml) $\times$ IRI (μU ml^{-1})/405.[19] Blood pressure was measured twice after 5 min of rest, with the subjects seated. Hypertension was defined as SBP $\geqslant 140$, DBP $\geqslant 90$ mm Hg or the current use of antihypertensive agents. Three hundred and ninety-five subjects were taking antihypertensive agents, and these subjects were included in the study. Individuals undergoing medical treatment and receiving diet therapy or exercise therapy for diabetes mellitus ($n=84$) were also included. Precise information on the types of antihypertensive agents or the nature of the treatment for diabetes was not obtained. All participants gave written informed consent to participate in the genetic analyses and in all other procedures associated with the study. The ethics committee of Osaka University approved the study protocol. The final number of subjects participating in the genetic study was 1488.

Genotyping

Genomic DNA was extracted from 200 μl of buffy coat using a QIAamp DNA Blood Kit (Qiagen, Hilden, Germany). We selected three SNPs, rs9939609, rs1121980 and rs1558902, in FTO, which were identified as being associated with obesity and/or diabetes in previous reports.[6,9,20,21] These SNPs were genotyped using TaqMan PCR methods with the following probes: C__30090620_10 for rs9939609, C__2031261_10 for rs1121980 and C__8917111_10 for rs1558902 (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Hardy–Weinberg equilibrium was calculated by a χ^2-test. Linkage disequilibrium was evaluated by SNP Alyze version 2.1 (DYNACOM Co., Ltd, Mohara, Japan). Associations between the polymorphisms and clinical variables were analyzed using one-way analysis of variance and analysis of covariance adjusted for confounding factors. Differences in genotype or allele distribution were examined by χ^2-analysis. All numerical values are expressed as mean ± s.d. Values of $P<0.05$ were considered to indicate statistical significance. To adjust for multiple testing of the three gene polymorphisms by Bonferroni's correction, we arbitrarily adopted $P<0.017$ ($=0.05/3$) as the level of statistical significance. Haplotype estimation was performed by the expectation-maximization algorithm. All analyses except analysis of covariance were performed with JMP statistical software (version 5; SAS Institute Inc., Cary, NC, USA), and analysis of covariance was performed with SPSSα statistical software (release 11.0.1; SPSS Inc., Chicago, IL, USA).

RESULTS

The total number of study subjects who were successfully genotyped for all three SNPs, rs9939609, rs1121980 and rs1558902 of FTO, was 1488. The characteristics of study subjects are shown in Table 1. In this cohort, the average BMI was much lower than that of study subjects in previous studies.[5,9,22,23] The prevalence of obesity defined as BMI $\geqslant 25$ kg m^{-2} according to Japanese criteria was 33%. DM, hypertension and MS by the Japanese definitions were exhibited by the study subjects at 7.8, 44.4 and 15.9%, respectively.

From the genotyping, the prevalences of each genotype in the three SNPs were determined to be AA/AT/TT=56/475/957 in rs9939609, AA/AG/GG=81/519/885 in rs1121980 and AA/AT/TT=59/468/959 in rs1558902. These allele frequencies are in accordance with Hardy–Weinberg equilibrium (data not shown).

The three SNPs, rs9939609, rs1121980 and rs1558902 in FTO, were tested for associations with hypertension, diabetes, dyslipidemia, obesity and MS in all the subjects using χ^2-tests. As shown in Table 2, there were no significant associations with hypertension or metabolic disorders in additive, dominant or recessive models. Table 3 shows blood pressure level and various metabolic parameters compared among genotypes for the three models of three SNPs in FTO using analysis of variance.

Additive and dominant models of rs1121980 showed significant differences in levels of IRI ($P=0.022$, 0.01, respectively) and HOMA-IR ($P=0.029$, 0.008, respectively), as shown in Table 3. Table 4 shows detailed data of IRI and HOMA-IR values composed among the genotypes in rs1121980. After adjusting for confounding factors including age, gender, BMI, abdominal circumference and presence of DM, there were still significant differences in IRI and HOMA-IR among genotypes in both additive and dominant models ($P=0.005$, 0.001, respectively) as determined by analysis of covariance.

Because some antihypertensive drugs might affect insulin sensitivity, we investigated the genotype comparison of HOMA-IR in both additive and dominant models for rs1121980 in subjects without hypertension ($n=740$). Significant differences were again identified: AA ($n=38$): 1.82 ± 4.04 vs. AG(223): 1.09 ± 0.84 vs. GG(418): 1.10 ± 0.94 in the additive model ($P=0.004$) and AA: 1.82 ± 4.04 vs. AG+GG(641): 1.10 ± 0.91 in the dominant model ($P=0.0009$).

Table 1 Characteristics of study subjects

	N=1488
Age (year)	62.7 ± 11.63
Gender (n, male/female)	M/F=582:906 (M: 39.1%)
BMI (kg m^{-2})	23.81 ± 3.24
Abdominal circumference (cm)	83.96 ± 10.20
Systolic blood pressure (mm Hg)	137.43 ± 22.79
Diastolic blood pressure (mm Hg)	76.37 ± 11.67
Total cholesterol (mg per 100 ml)	201.18 ± 31.64
HDL-CHO (mg per 100 ml)	50.59 ± 12.28
LDL-CHO (mg per 100 ml)	130.04 ± 29.62
Triglyceride (mg per 100 ml)	102.72 ± 57.86
FBS (mg per 100 ml)	97.8 ± 24.83
IRI (μU ml^{-1})	5.203 ± 3.71
HOMA-IR	1.32 ± 1.43
	(mean ± s.d.)

Abbreviations: BMI, body mass index; FBS, fasting blood sugar; HDL-CHO, high-density-lipoprotein cholesterol; IRI, immunoreactive insulin; LDL-CHO, low-density-lipoprotein cholesterol; HOMA-IR, homeostasis model assessment insulin resistance. HOMA-IR=(FBS×fasting IRI)/405.

Table 2 Relationships between three SNPs in *FTO* and metabolic diseases

	rs9939609			*rs1121980*			*rs1558902*		
	Additive model	Dominant model AA vs. AT+TT	Recessive model TT vs. AT+AA	Additive model	Dominant model AA vs. AG+GG	Recessive model GG vs. AG+AA	Additive model	Dominant model AA vs. AT+TT	Recessive model TT vs. AT+AA
Dyslipidemia	0.5628	0.729	0.2845	0.5386	0.3025	0.4837	0.7339	0.5975	0.4805
Diabetes	0.4729	0.4982	0.4221	0.5166	0.3312	0.7613	0.4923	0.4362	0.514
Hypertension	0.5142	0.3994	0.3291	0.6366	0.9944	0.3623	0.5557	0.6346	0.2859
Obesity	0.9303	0.9001	0.7057	0.8787	0.9718	0.6202	0.8242	0.9073	0.5357
Treatment history									
Dyslipidemia	0.86	0.8151	0.6768	0.6608	0.5948	0.3888	0.8033	0.9036	0.5535
Diabetes	0.4843	0.2792	0.822	0.403	0.4852	0.3648	0.4943	0.3398	0.677
Hypertension	0.4274	0.2045	0.5304	0.6523	0.3601	0.6956	0.4885	0.3156	0.3666
Metabolic syndrome	0.7067	0.9954	0.4213	0.6112	0.9917	0.3415	0.6153	0.8625	0.3767

Values are indicated as *P*-values.

Table 3 Relationships between three SNPs in *FTO* and metabolic parameters

	rs9939609			*rs1121980*			*rs1558902*		
	Additive model	Dominant model AA vs. AT+TT	Recessive model TT vs. AT+AA	Additive model	Dominant model AA vs. AG+GG	Recessive model GG vs. AG+AA	Additive model	Dominant model AA vs. AT+TT	Recessive model TT vs. AT+AA
T-CHO (mg per 100 ml)	0.1799	0.6608	0.0641	0.3366	0.351	0.1719	0.3976	0.7541	0.1748
HDL-CHO (mg per 100 ml)	0.4853	0.5142	0.4232	0.8663	0.948	0.6243	0.5043	0.4449	0.5204
LDL-CHO (mg per 100 ml)	0.2946	0.5909	0.1191	0.5649	0.4123	0.3708	0.4825	0.7386	0.2273
TG (mg per 100 ml)	0.872	0.6059	0.8236	0.6308	0.6027	0.3557	0.6878	0.4164	0.9499
FBS (mg per 100 ml)	0.9072	0.7465	0.7072	0.9321	0.7166	0.9876	0.9539	0.8355	0.7836
IRI (μU ml^{-1})	0.3919	0.2543	0.3002	0.022	0.01	0.0876	0.2408	0.311	0.1145
HOMA-IR	0.7414	0.4844	0.6122	0.0294	0.008	0.3577	0.6697	0.5585	0.4156
SBP (mm Hg)	0.7555	0.6449	0.4893	0.3885	0.8893	0.1772	0.6958	0.9078	0.3991
DBP (mm Hg)	0.9621	0.9567	0.7814	0.3831	0.8359	0.2113	0.7915	0.6504	0.7127
BMI	0.6663	0.9483	0.3768	0.4627	0.9628	0.23	0.3762	0.9994	0.1787

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; FBS, fasting blood sugar; HDL-CHO, high-density-lipoprotein cholesterol; HOMA-IR, homeostasis model assessment insulin resistance; IRI, immunoreactive insulin; LDL-CHO, low-density-lipoprotein cholesterol; SBP, systolic blood pressure; T-CHO, total cholesterol; TG, triglyceride.
Values are indicated as *P*-values.

There was strong linkage disequilibrium among the three SNPs in *FTO*. The r^2 values were 0.811 between rs9939609 and rs1121980, 0.956 between rs9939609 and rs1558902 and 0.821 between rs1121980 and rs1558902. Table 5 shows the results of comparison for metabolic parameters for each haplotype of the three SNPs in additive, dominant and recessive models analyzed by analysis of variance. Low-frequency haplotypes, namely, those with frequencies below 1%, were excluded from analysis. Five haplotypes were analyzed. H2 had a strong association with insulin resistance (Table 5), although it was present at a low frequency ($n=21$).

DISCUSSION

This study is the first reported investigation of the association between genetic variations in *FTO* and detailed metabolic parameters in the Japanese general population. We selected three SNPs, rs9939609, rs1121980 and rs1558902 in *FTO*, that had been found to be strongly associated with obesity, defined by BMI, in genome-wide association

Table 4 Detailed data of IRI and HOMA-IR values compared among genotypes in rs1121980

SNP rs1121980	Genotype group	HOMA-IR	P-value	IRI (μU ml^{-1})	P-value
	GG (828)	1.29 ± 1.42		5.06 ± 3.38	
	AG (482)	1.30 ± 1.02	0.006	5.27 ± 3.67	0.020
	AA (76)	1.74 ± 2.99		6.26 ± 6.34	
	GG	1.29 ± 1.42	0.794	5.05 ± 3.38	0.421
	AG+AA	1.36 ± 1.46		5.41 ± 4.15	
	GG+AG	1.29 ± 1.29	0.002	5.14 ± 3.49	0.013
	AA	1.73 ± 2.99		6.26 ± 6.34	

Abbreviations: HOMA-IR, homeostasis model assessment insulin resistance; IRI, immunoreactive insulin; SNP, single nucleotide polymorphism.
Values are shown as mean ± s.d.
Subjects with data of HOMA-IR and IRI were analyzed in Table 4.

Table 5 The results of comparison between haplotypes with three SNPs for metabolic parameters

	rs9939609/rs1121980/rs1558902	T-CHO	HDL-CHO	LDL-CHO	IRI	HOMA-IR
H1	AA/AA/AA (56) vs. others	0.6608	0.5142	0.5909	0.2543	0.4844
H2	AT/AA/AT (21) vs. others	0.1853	0.2637	0.3121	0.0006[a]	<0.0001[b]
H3	AT/AG/AT (441) vs. others	0.2977	0.5497	0.3463	0.9984	0.4927
H4	TT/AG/TT (67) vs. others	0.8063	0.4806	0.4044	0.5983	0.7029
H5	TT/GG/TT (878) vs. others	0.1305	0.5129	0.3147	0.1002	0.39

Abbreviations: HDL-CHO, high-density-lipoprotein cholesterol; HOMA-IR, homeostasis model assessment insulin resistance; IRI, immunoreactive insulin; LDL-CHO, low-density-lipoprotein cholesterol; T-CHO, total cholesterol.
Values are indicated as P-values.
[a]AT/AA/AT 8.11±9.89 (N=19). Others 5.16±3.54 (N=1370).
[b]AT/AA/AT 2.72±5.59 (N=19). Others 1.30±1.28 (N=1370).

studies in various ethnicities.[22,24-29] In Japan, Hotta et al.[9] reported that rs1558902 in FTO was most significantly associated with obesity in a case–control association study using severely obese Japanese subjects (average BMI⩾30 kg m^{-2}).[9] In this study, we investigated the association between various metabolic parameters including hypertension, DM, obesity and MS in subjects participating in the Tanno–Sobetsu cohort study, a study of a Japanese representative rural cohort in Hokkaido. The average BMI of the study subjects was 23.81 ± 3.24 kg m^{-2}, which is close to the national average in Japan. In this study, none of the three SNPs was associated with obesity, defined by BMI, higher abdominal circumference or prevalence of MS, defined by Japanese criteria. In addition, none of the three SNPs was associated with hypertension, dyslipidemia or prevalence of DM.

Only one SNP, rs1121980, showed a strong correlation with HOMA-IR, which is an index of insulin resistance, in additive and dominant models. Subjects with AA in rs1121980 had a much higher HOMA-IR and a higher insulin resistance than subjects without the AA genotype (P=0.008). This P-value is considered significant (P<0.017) after Bonferroni's correction to adjust for multiple testing of the three SNPs. Subjects with the haplotype H2, which includes AA in rs1121980, had a higher HOMA-IR than other subjects (Table 5). Thus, we conclude that rs1121980 in FTO is associated with insulin resistance in the Japanese general population. Because a recent report indicates that a gain of function of FTO induces insulin resistance,[7] rs1121980 located in an intron may regulate FTO gene function by affecting splicing variation. After adjusting for obesity and the prevalence of DM, rs1121980 is independently associated with insulin resistance. Therefore, rs1121980 may affect insulin resistance, directly and not only indirectly by obesity.

In this study, three SNPs in FTO were not associated with obesity. Several reasons for this are considered. One is the difference between Caucasian and Japanese general populations in the severity of obesity. In fact, SNPs in FTO were associated with obesity in a study using Japanese subjects with severe obesity (average BMI⩾30 kg m^{-2}).[9] Another reason is the differences in allele frequency among FTO SNPs. In the cases of rs9939609, allele frequency information obtained from HapMap database (http://hapmap.ncbi.nlm.nih.gov/) shows significant differences between Caucasian (AA/AT/TT=0.117/0.667/0.217) and Japanese populations (0.067/0.200/0.733).

In summary, an SNP located in an intron, rs1121980, and a haplotype of three SNPs in FTO that includes this SNP, may be important in the progression of insulin resistance in Japanese subjects. This SNP may be an independent risk factor for future MS, hypertension and DM in Japanese subjects. However, this study has limitations because of its cross-sectional design. Prospective studies investigating the relationship between these SNPs and the development of MS, hypertension and DM over a long time scale are necessary.

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Brief Communication

A haplotype of the CYP4F2 gene associated with myocardial infarction in Japanese men

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ABSTRACT

This study assessed associations between the CYP4F2 gene and myocardial infarction (MI), using a haplotype-based case-control study of 234 MI patients and 248 controls genotyped for 5 single-nucleotide polymorphisms (rs3093105, rs3093135, rs1558139, rs2108622, rs3093200). For men, G allele frequency of rs2108622 and frequency of the T-C-G haplotype were significantly higher, and frequency of the T-C-A haplotype was significantly lower for MI patients than for controls ($P = 0.006$, $P = 0.001$ and $P = 0.002$, respectively).

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Introduction

Myocardial infarction (MI) is thought to be a complex multifactorial and polygenic disorder [1,2]. Accumulating evidence has demonstrated that cytochrome p450 (CYP) enzymes are involved in the pathogenesis of the risk of MI [3]. CYP1A1, CYP1A2 [4,5], CYP2C8, CYP2C9, CYP2J2 [6–8], CYP8A [9], CYP11B2 [10], and CYP17 and CYP19 genes have been shown to be associated with MI [11]. The CYP4F2 subfamily is expressed in liver, heart, lung, renal tissues and white blood cells, and is involved in the metabolism of both leukotriene B_4 (LTB_4) and 20-hydroxyeicosatetraenoic acid (20-HETE) [12], the latter of which plays a crucial role in the modulation of cardiovascular homeostasis [12]. The aim of this study was to assess associations between the human CYP4F2 gene and MI via a haplotype-based case-control study.

Subjects and methods

Subjects

Subjects diagnosed with MI ($n = 234$, M/F = 189/45) were recruited at Nihon University Itabashi Hospital and at other neighboring hospitals in Tokyo. Patient age ranged from 40 to 85 years (mean ± standard deviation [SD], 61.8 ± 9.3 years). Controls were all Japanese ($n = 248$, M/F = 124/124), members of the New Elder Citizen Movement in Japan, and living in Tokyo or its surrounding suburbs. Control age ranged from 66 to 89 years (mean ± SD, 77.6 ± 3.6 years). Clinical profiles of the MI patients and controls have been described in previous reports [9,13,14]. Informed consent was obtained from each subject in accordance with the protocol approved by the Human Studies Committee of Nihon University.

Genotyping

A total of 225 single-nucleotide polymorphisms (SNPs) for the human CYP4F2 gene are listed in the National Center for Biotechnology Information SNP database Build 126 (http://www.ncbi.nlm.nih.gov/SNP). We screened data for Tag SNPs on the International HapMap Project website (http://www.hapmap.

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org/index.html.ja) with a cut-off level of $r^2 \geqslant 0.5$ and a cut-off level of minor allele frequency (MAF) $\leqslant 0.1$. According to the above criteria, we selected rs3093135 (SNP2, C_27482167_10), rs1558139 (SNP3, C_2583813_10), rs2108622 (SNP4, C_16179493_40) from this gene. Fundamentally, these three SNPs were thought to be sufficient as genetic markers for the haplotype-based case-control study. Both rs3093135 and rs1558139 are located in the intron, while rs2108622 is located in the exon and known as a nonsynonymous substitution. A variation of rs2108622 (V433M) results in decreased 20-HETE production from arachidonic acid [17]. SNPs located in introns are available as genetic markers [16]. Furthermore, we selected rs3093105 (SNP1) and rs3093200 (SNP5), which are known as nonsynonymous SNPs, because nonsynonymous SNPs yield amino acid changes. Nosynonymous SNPs can be not only functional mutations, but also genetic markers.

Genotyping and biochemical analysis were performed as described previously [13].

Statistical analysis

All continuous variables were expressed as mean ± SD. All statistical analyses were performed as described previously [13]. No cases of C/A heterozygous or A/A homozygous of SNP5 were found in both MI patients and control subjects, so SNP5 was unavailable for the haplotype-based case-control study.

Results and discussion

Mean age was significantly higher in controls than in the MI group. For the total population and male and female subjects, heart rate and prevalences of diabetes mellitus and smoking were significantly higher for MI patients than controls. For the total population and male subjects, body mass index and incidence of hyperlipidemia were significantly higher in MI patients. For the total population and female subjects, plasma concentration of total cholesterol and serum concentration of creatinine differed significantly between MI patients and controls. For the total population, systolic blood pressure and prevalence of hypertension were significantly higher for MI patients than for controls. No significant differences existed in systolic blood pressure or prevalence of drinking between MI patients and controls.

Genotype distributions of each SNP in controls agreed with predicted Hardy–Weinberg equilibrium values (data not shown). For males, the G allele of SNP4 (rs2108622) was significantly higher in MI patients than in controls ($P = 0.006$). Only the CC homozy-

gous genotype of SNP5 was found in both MI patients and control subjects, so SNP5 was not used in haplotype-based case-control analysis.

In linkage disequilibrium analysis, all four SNPs were located in 1 haplotype block for $|D'| > 0.25$. The r^2 of SNP1-SNP2 was >0.5, which means that SNP1 and SNP2 could not be used to construct haplotypes simultaneously. As the minor allele frequency of SNP2 was larger than that of SNP1, we constructed haplotypes using SNP2, SNP3, and SNP4. In haplotype-based case-control analysis, for men, the overall distribution of haplotypes differed significantly between MI patients and controls ($P = 0.001$, Table 1). Also, for men, frequency of the H1 haplotype was significantly higher for MI patients than for controls ($P = 0.001$), and frequency of the H5 haplotype was significantly lower for MI patients than for controls ($P = 0.002$).

Stec et al. recently identified a guanine-to-adenine polymorphism (rs2108622, V433M) in the CYP4F2 gene associated with a reduction in 20-HETE from AA in vitro [15]. Ward et al. demonstrated that the CYP4F2 GA/AA genotype is significantly associated with an increase in both 20-HETE excretion and systolic blood pressure [17]. On the point of whether the A allele is associated with a reduction or increase in 20-HETE, these results are in conflict. This is possibly due to the different study methods, with one an in vitro study, and the other an in vivo study. Fava et al. [18] showed that for men, M433 carriers showed significantly higher blood pressure than V433V homozygotes, similar to the results of Ward et al. [17]. Furthermore, for men, M433 carriers showed a significantly higher incidence of ischemic stroke than V433V homozygotes [18]. We identified a significantly higher frequency of the G allele of SNP4 (rs2108622) for MI patients than for controls among Japanese men, meaning the risk of MI is increased in men carrying the G allele of SNP4. Our results differ from those of Ward et al. [17] and Fava et al. [18], who found the A allele as a risk factor for hypertension or ischemic stroke. These conflicting results may reflect the complex function of 20-HETE, which blocks sodium transport and acts as a natriuretic in the renal tubule, but acts as a vasoconstrictor in the renal vasculature.

Our previous haplotype-based case-control study identified susceptibility genes for multifactorial diseases using SNPs located in introns as genetic markers [14]. Based on such findings, we hypothesized that haplotype analysis would be useful for the assessment of associations between haplotypes and MI. For men, we succeeded in identifying a susceptibity haplotype (T-C-G, SNP2-SNP3-SNP4) and a protective haplotype (T-C-A, SNP2-SNP3-SNP4), and these haplotypic analysis results are consistent

Table 1
Haplotype analysis in patients with MI and control subjects.

CYP4F2 polymorphism			Overall P			Frequency in total		P	Frequency in men		P	Frequency in women		P
	SNP2	SNP3	SNP4	Total	Man	Women	MI patients	Controls		MI patients	Controls		MI patients	Controls	
Haplotypes	SNP2	SNP3	SNP4	0.223	0.001	0.277									
H1	Mj	Mj	Mj				0.595	0.530	0.047	0.610	0.467	0.001*	0.534	0.563	0.646
	T	C	G												
H2	Mj	Mn	Mj				0.169	0.189	0.491	0.163	0.197	0.284	0.193	0.164	0.513
	T	T	G												
H3	Mn	Mj	Mn				0.084	0.110	0.183	0.088	0.119	0.216	0.068	0.102	0.376
	A	C	A												
H4	Mj	Mn	Mn				0.152	0.173	0.372	0.139	0.193	0.076	0.205	0.144	0.201
	T	T	A												
H5	Mj	Mj	Mn				—	—	—	0.000	0.024	0.002*	—	—	—
	T	C	A												
H6	Mn	Mj	Mj				—	—	—	—	—	—	0.000	0.027	0.105
	A	C	G												

MI, myocardial infarction; Mj, major; Mn, minor; Haplotypes with frequency >0.02 were estimated using SNPAlyze software. P value was calculated by chi-square analysis.
* $P < 0.01$.

with the genotypic analysis results of SNP4 that the G allele confers risk and the A allele is protective.

In conclusion, the G allele of rs2108622 can offer a genetic marker for MI in Japanese men and the T-C-G haplotype of the human CYP4F2 gene appears to represent a genetic marker of MI in Japanese men.

Acknowledgments

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Atherogenic dyslipidemia and altered hepatic gene expression in SHRSP.Z-Lepr^fa/IzmDmcr rats

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Abstract. We investigated lipid and lipoprotein abnormalities in SHRSP fatty rats as a new animal model of metabolic syndrome. We examined differentially expressed genes in the liver, one of the major tissues contributing to lipid metabolism. Using gel filtration high performance liquid chromatography, increased cholesterol concentrations of small particle size low-density lipoprotein (LDL) fractions were observed in SHRSP fatty rats, whereas the Zucker Fatty strain did not show a similar elevation of cholesterol content. Existence of apolipoprotein B in these fractions was confirmed by Western blotting. The small particle size of the LDL fractions was significantly decreased by a 4-week fenofibrate treatment. Microarray analysis identified seventeen genes that were significantly upregulated and ten that were significantly decreased in liver tissues of SHRSP fatty rats compared with levels in SHRSP rats. Stearoyl-coenzyme A desaturase 1, fatty acid synthase, ATP citrate lyase, and sterol regulatory element binding factor 1 genes were among the upregulated genes. These findings suggest that SHRSP fatty rats carry small dense LDL like particles which is a common lipid abnormality in the metabolic syndrome. Three of ten genes upregulated in liver tissues of

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SHRSP fatty rats play a role in this metabolic abnormality and are a therapeutic target of this metabolic syndrome.

Introduction

Metabolic syndrome is characterized by a constellation of cardiovascular risk factors, including atherogenic dyslipidemia, abnormal glucose tolerance, hypertension, and visceral obesity, which are intimately associated with insulin resistance and hyperinsulinemia (1-3). The dyslipidemia of metabolic syndrome features hypertriglyceridemia involving elevated concentrations of triglyceride rich lipoproteins, subnormal levels of high-density lipoprotein (HDL) choresterol, or both. Major quantitative modifications of the atherogenic lipid profile typically include a small, dense, low-density lipoprotein (sdLDL) phenotype (4-6). Retrospective studies provided evidence that the preponderance of sdLDL particles is associated with an increased cardiovascular risk, a suggestion that was later also supported by a prospective study (7). These findings imply that the evaluation of sdLDL cholesterol levels enhances the prediction of cardiovascular events (8,9).

Hiraoka-Yamamoto *et al* (10) established a new rat model of metabolic syndrome, SHRSP.Z-Lepr^fa/IzmDmcr, by crossing SHRSP rats of the Izumo strain (a genetic model of severe hypertension) with Zucker Fatty (ZF) rats. SHRSP fatty rats carry the leptin receptor *OB-Rb* gene mutation found in ZF rats and become obese while developing hypertension. We previously reported that SHRSP fatty rats exhibit obesity and hypertension accompanied by hypertrophy of the midlayer smooth muscle of the arteries, increased non-fasting triglyceride levels and increased insulin resistance. Therefore, we determined that the phenotype of SHRSP fatty rats is similar to that of human metabolic syndrome and is a useful tool for investigating the molecular mechanisms underlying metabolic syndrome (11).

Fibric acid derivatives (fibrates) are lipid-lowering drugs which selectively target therapeutic goals for individuals with features of metabolic syndrome. One specific fibrate, fenofibrate, was effective in normalizing lipoprotein levels and reducing insulin resistance in patients with metabolic syndrome (12,13). Additionally, plasma triglyceride lowering agents enlarge the LDL size (14). However, few studies examine the quantitative change of LDL sub-species by fibrate treatment. One of these studies, by Takuno *et al* (15), reported that fenofibrate decreased sdLDL specifically, without lowering total LDL levels.

The liver is a major determinant of the whole body fatty acid and neutral lipid metabolism as well as circulating levels of atherogenic apolipoprotein B containing lipoproteins (16-18). A marked increase in the production of apolipoprotein B containing lipoprotein in the liver is frequently associated with metabolic syndrome (19). In fact, Adiels *et al* reported that overproduction of very low density lipoprotein (VLDL) particles is driven by the amount of fat accumulated in the liver (20). However, underlying mechanisms for this overproduction of fatty liver related lipids is still unclear.

In the current study, to clarify the mechanism of such lipid abnormalities in metabolic syndrome, we examined the plasma lipoprotein profile, evaluated the effect of fenofibrate, and examined the liver gene expression profile in SHRSP fatty rats.

Materials and methods

Animal subjects. Our study conforms to the guidelines published in the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Wistar-Kyoto/Izumo (WKY) and SHRSP/Izumo rats were obtained from Disease Model Cooperative Research Association (Kyoto, Japan). ZF rats were obtained from Tokyo Experimental Animal (Tokyo, Japan). SHRSP fatty were obtained by crossing SHRSP/Izumo with ZF rats as described previously (10). Four of each strain of rats were fed standard rat chow and then fasted for 12 h before blood collection.

Fenofibrate (100 mg/kg) (Sigma-Aldrich, Irvine, UK) was dissolved/suspended in 0.25% methylcellulose (Wako, Osaka, Japan) at a concentration that would allow daily doses of 10 ml suspended compounds. After a 4-week fenofibrate treatment of four SHRSP fatty rats, the animals were euthanized by diethyl ether and blood was collected by cardiac puncture.

Histologic examination. Twenty-four week-old WKY, SHRSP, ZF, and SHRSP fatty rats were anesthetized with sodium pentobarbital (Nembutal, 50 mg/kg IP; DS Pharma Biomedical, Osaka, Japan) and perfused with saline followed by 10% neutral buffered formalin. The liver was removed, tissue specimens were embedded in paraffin, and 2-μm thick slices were stained with hematoxylin-eosin. These procedures were performed by a pathologist with no prior knowledge of the experimental groups.

High-performance liquid chromatography (HPLC). Plasma lipoprotein profiles were analyzed by HPLC using gel-permeation column(s) (Lipopropak XL; 7.8 mm x 300 mm; Tosoh, Tokyo, Japan) with 0.05 M Tris-buffered acetate, pH 8.0, containing 0.3 M sodium acetate, 0.05% sodium azide, and 0.005% Brij-35 at a flow rate of 0.7 ml/min and an online enzymatic lipid-detection system (21-23).

Western blot analysis for apolipoprotein B and AI proteins. Fractionated samples were incubated with equal amount of lysis buffer (6% SDS, 40% glycerol, 0.5% bromophenol blue) at 95°C for 5 min, subjected to 2-15% SDS-polyacrylamide gel (Daiichi Chemical, Tokyo, Japan) electrophoresis, and electro-blotted onto PVDF membranes (GE Healthcare Biosciences, Piscataway, NJ). Blots were incubated with goat polyclonal antibody specific for anti-apolipoprotein B or rabbit polyclonal antibody specific for apolipoprotein AI (Santa Cruz Biotechnology, Santa Cruz, CA) as primary antibodies, and then with rabbit anti-goat IgG (Cappel, West Chester, PA) or goat anti-rabbit IgG (RPN2124, GE Healthcare), as secondary antibodies. Bound antibodies were detected by enhanced chemi-luminescence (RPN2124, GE Healthcare) and scanned by lumino-image analyzer (LAS-3000, Fuji film, Tokyo, Japan).

DNA microarray procedure. Total RNA was obtained from liver tissue of SHRSP fatty and SHRSP rats by successive extractions with Trizol and RNeasy Mini Kits (Qiagen, Valencia, CA). The RNA was assessed for quality and quantity with a Bioanalyzer (Agilent, Palo Alto, CA). DNA microarray analysis was performed according to the manufacturer's instructions (Affimetrix, Santa Clara, CA). In brief, double-stranded cDNA was synthesized from 10 μg of total RNA by reverse transcription with SuperScript Choice System (Invitrogen, Carlsbad, CA). Biotinylated cRNA was transcribed from the double-stranded cDNA by T7 RNA polymerase reaction with an RNA Transcript Labeling Kit (Enzo Biochem, Framingdale, NY), fragmented, and applied to Gene Chips (Rat Genome 230 2.0 Array, Affimetrix). After hybridization for 16 h at 45°C, the Gene Chip was washed and labeled with R-phycoerythrin streptavidin using the Affymetrix Fluidics Station 400. The fluorescent signal intensities were measured with an Affymetrix Scanner. Raw data were extracted with Microarray Suite 5 (Affymetrix) and analyzed with GeneSpring GX (Agilent). Values below 0.01 were set to 0.01, and each measurement was divided by the 50th percentile of all measurements in that sample. Each gene was divided by the median of its measurements in all samples. If the median of the raw values was <10 then each measurement for that gene was divided by 10 if the numerator was >10, otherwise the measurement was disregarded. Results were expressed as a fold change of the mean of four SHRSP fatty and four SHRSP rats.

Statistical analysis. Results are given as the mean ±SEM. The significance of differences between the mean values was evaluated by Student's t-test for unpaired data. Differences of P<0.05 were considered significant.

Results

Body weight, plasma lipid, and lipoprotein profile. Body weight of SHRSP fatty rats was significantly higher compared to WKY rat strain, and almost comparable with the ZF strain (Table IA). Plasma total cholesterol level and cholesterol levels in each major lipoprotein fraction are shown in Table IB. Plasma total cholesterol level in SHRSP fatty rats (181.2±8.4 mg/dl) was significantly higher than that in each parental strain (p<0.05). Whereas no significant difference was observed in LDL fraction

Table I. Plasma total cholesterol and triglyceride concentrations of each fraction of SHRSP fatty, ZF, and SHRSP rats.

A, Body weight (grams)

SHRSP fatty	446.0±22.0[a]
ZF	452.0±8.0[a]
SHRSP	262.0±4.9

B, Total cholesterol

	Total	CM (>80 nm)	VLDL (30-80 nm)	LDL (16-30 nm)	HDL (8-16 nm)
SHRSP fatty	181.1±8.4[a]	0.2±0.0	48.6±3.2[a]	31.3±3.6[a]	101.0±4.1[a]
ZF	97.8±3.4	0.0±0.0	9.7±0.5	8.3±0.7	79.8±2.3
SHRSP	67.9±2.0	0.0±0.0	2.5±0.6	9.0±0.3	56.4±2.7

C, Triglycerides

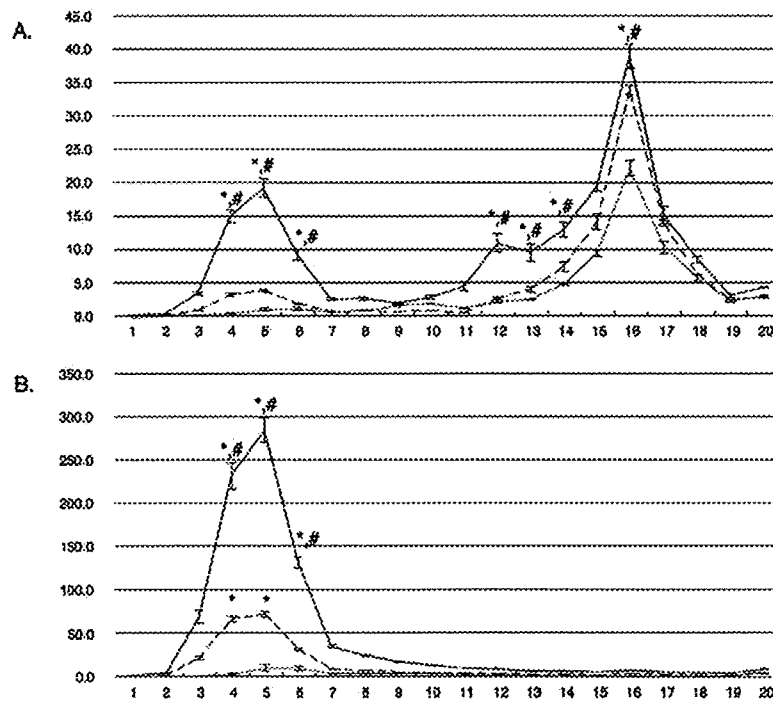|					
SHRSP fatty	862.7±43.6[a]	4.4±0.5	747.7±42.5[a]	74.0±1.2[a]	36.5±0.8[a]
ZF	231.0±10.2[a]	0.8±0.1	196.6±8.8[a]	21.2±1.0	12.5±1.1
SHRSP	39.2±8.6	0.1±0.0	23.7±7.7	11.6±0.8	3.8±0.3

mg/dl; [a]$p<0.05$ vs SHRSP.

Figure 1. Lipoprotein profile of each rat strain determined by high performance liquid chromatography. (A) Elution pattern of HPLC monitored by cholesterol concentration in each fraction. (B) Elution pattern of HPLC monitored by triglyceride concentration in each fraction. Black solid line, lipid concentration in each fraction from SHRSP fatty; dashed line, lipid concentration in each fraction from ZF; solid line, lipid concentration in each fraction from SHRSP.

cholesterol levels, cholesterol level of HDL fraction in SHRSP fatty rats was significantly higher (101.01±4.05 mg/dl) than in each parental strain (p<0.05) (Table IB). Plasma triglyceride levels in SHRSP fatty rats were significantly higher

(862.7±43.6 mg/dl) compared to the parental strains (p<0.05), and in SHRSP fatty rats, about 86.7% of plasma triglycerides were included in the VLDL fraction (747.7±42.5 mg/dl) (Table IC).
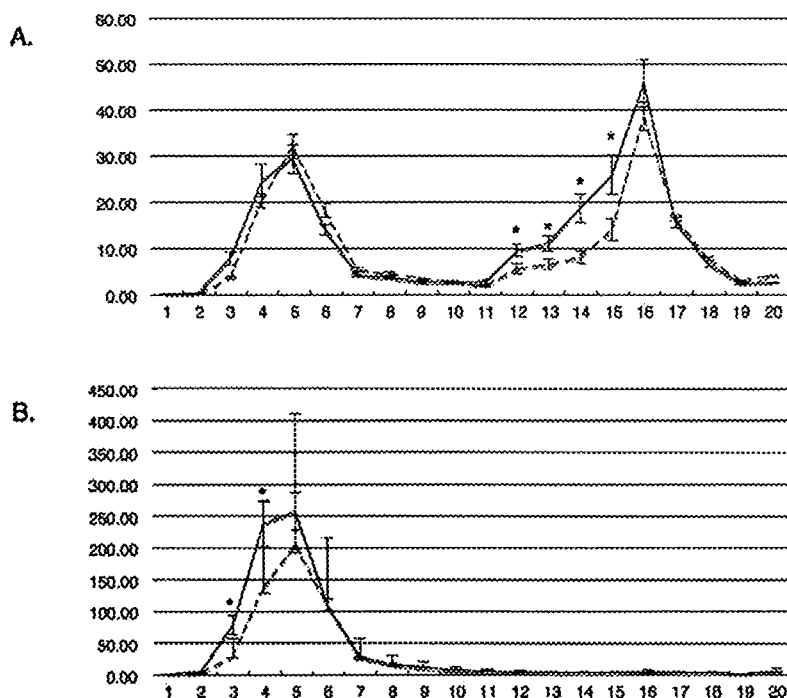
Figure 2. Effect of fenofibrate administration on lipoprotein profile of SHRSP fatty determined by high performance liquid chromatography. (A) Elution pattern of HPLC monitored by cholesterol concentration in each fraction. (B) Elution pattern of HPLC monitored by triglyceride concentration in each fraction. Solid line, lipid concentration of each fraction before fenofibrate treatment; dashed line, lipid concentration in each fraction after fenofibrate treatment.
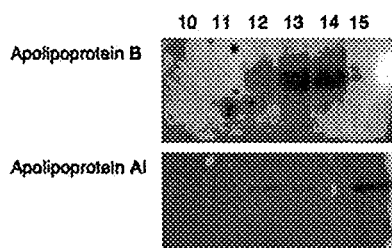

Figure 3. Western blot analysis for apolipoprotein B and AI proteins in samples from HPLC fractions. Fraction numbers 10 to 15 are identical to fraction numbers in Figs. 1 and 2.

Lipoprotein profile of each rat strain determined by HPLC. To determine the lipoprotein profile of each rat strain, gel filtrated HPLC was performed and cholesterol (Fig. 1A) and triglyceride (Fig. 1B) levels in each fraction were evaluated. In VLDL fractions (fractions 3-7), cholesterol and triglyceride levels in SHRSP fatty rats were significantly higher than in each parental strain. In fractions 12, 13, and 14, significantly higher cholesterol levels were also observed in SHRSP fatty rats, indicating the existence of very small particle size LDLs. In fraction 16, cholesterol levels were significantly lower in SHRSP rats compared with SHRSP fatty or ZF rats.

Western blot analysis for apolipoprotein B and AI proteins. To examine the feature of lipoproteins included in fractions 12, 13, and 14, we performed Western blot analysis for apolipoprotein

B and AI on each fraction from SHRSP fatty rats. Apolipoprotein B was detected by Western blot analysis in fractions 12, 13, and 14, indicating the existence of apolipoprotein B containing small particle size lipoprotein (Fig. 3). Apolipoprotein AI was strongly stained in the sample from fraction 15, indicating the existence of large particle size HDL (Fig. 3).

Effect of fenofibrate on lipid and lipoprotein profiles of SHRSP fatty rats. Plasma lipid and lipoprotein profile before and after fenofibrate administration are shown in Table II. Plasma triglyceride levels were significantly reduced after 4 weeks of fenofibrate administration. VLDL levels also showed a tendency to lower after fenofibrate treatment. However, the results were not statistically significant.

The effect of fenofibrate on the plasma lipoprotein profile was evaluated by HPLC. Cholesterol levels in fractions 12, 13, and 14 were significantly reduced after fenofibrate treatment and triglyceride levels in fractions 3 and 4 were significantly reduced after fenofibrate treatment.

Pathologic liver findings in SHRSP fatty, SHRSP, and ZF rats. The typical hematoxylin-eosin staining results obtained upon histological examinations are shown in Fig. 4. The liver in SHRSP fatty and ZF rats showed marked lipid accumulation compared to that of SHRSP rats.

Gene expression profiling of liver tissue by DNA microarray. To identify the genes differentially expressed (more than 3-fold) between SHRSP fatty and SHRSP rats, we carried out microarray analysis. Seventeen genes were significantly up-

Table II. Plasma total cholesterol and triglyceride concentrations before and after fenofibrate administration.

	Total	CM (>80 nm)	VLDL (30-80 nm)	LDL (16-30 nm)	HDL (8-16 nm)
Total cholesterol					
Before	227.7±18.8	0.31±0.1	79.3±9.2	31.4±3.7	116.7±13.9
After	194.3±15.2	0.2±0.0	79.1±6.2	24.0±3.0	91.1±7.7
Triglycerides					
Before	777.0±99.8[a]	5.2±1.1	707.2±90.4	45.6±6.2	19.0±2.3
After	566.3±36.6	1.2±0.2	507.5±33.5	40.2±2.7	17.5±0.7

mg/dl; [a]$p<0.05$ vs SHRSP.

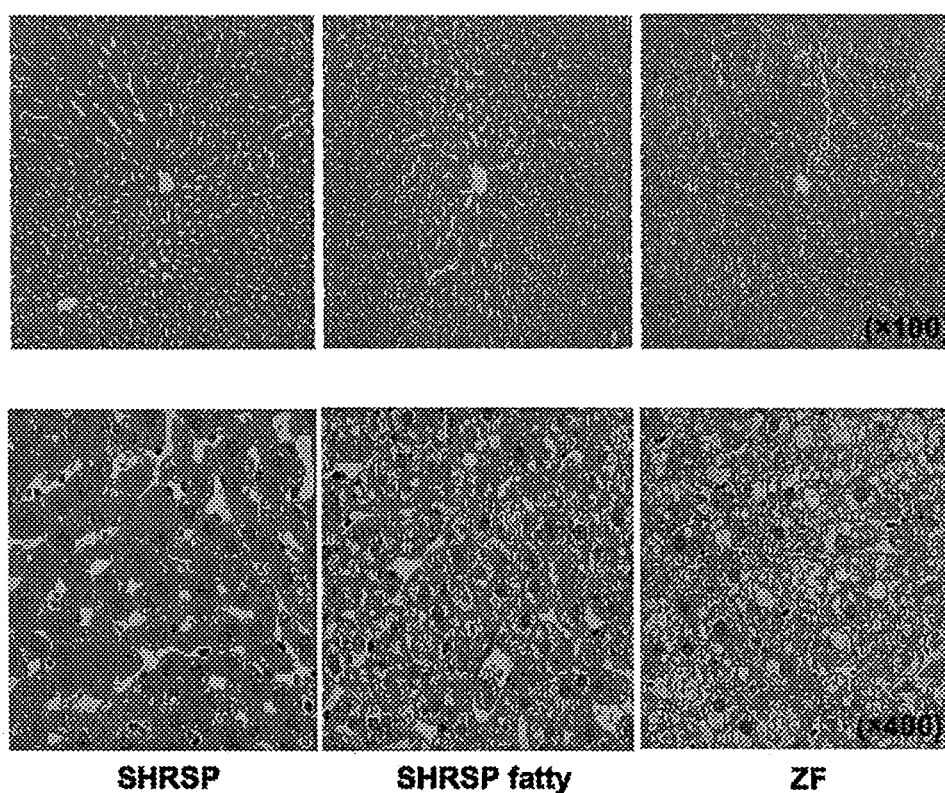**SHRSP**　　　**SHRSP fatty**　　　**ZF**

Figure 4. Pathologic findings of liver in SHRSP, SHRSP fatty, and ZF rats.

regulated in liver tissue in SHRSP fatty rats compared to SHRSP rats, including stearoyl-coenzyme A desaturase 1, fatty acid synthase, alcohol dehydrogenase 1, glucokinase, and sterol regulatory element binding factor 1.

Ten genes were significantly reduced (less than 0.3-fold) in the liver tissue of SHRSP fatty rats compared with SHRSP rats, inlcuding Fatty acid desaturase 1, ATP-binding cassette, subfamily C, member 2, solute carrier organic anion transporter family, member 1b2, and Hydroxysteroid (17-ß) dehydrogenase 2. Results for all the differentially expressed genes are in Table III. Changes in gene expression in SHRSP fatty rats compared with the control strains discovered by microarray analysis were further confirmed by RT-PCR.

Discussion

In this study, we investigated lipid and lipoprotein abnormalities in SHRSP fatty rats as a new animal model of metabolic syndrome and examined the differentially expressed genes in the liver, since the liver is one of the major contributors to lipid metabolism.

In a previous study, we found that SHRSP fatty rats exhibited obesity and hypertension accompanied by hypertrophy of the midlayer smooth muscle of the arterioles, increased non-fasting triglyceride levels, and increased insulin resistance. Additionally, we determined that the phenotype of SHRSP fatty rats is similar to that of human metabolic

Table III. Genes differentially expressed in liver tissue of SHR-SP fatty and SHRSP.

Fold change	Description
Upregulated genes	
25.66[a]	Stearoyl-coenzyme A desaturase 1
11.77[a]	Fatty acid synthase
5.32[a]	Thyroid hormone responsive protein
4.56[a]	Ethylmalonic encephalopathy 1
4.52[a]	Alcohol dehydrogenase 1
3.89[a]	Flavin containing monooxygenase 5
3.79[a]	Glucokinase
3.51[a]	Rat senescence marker protein 2A gene, exons 1 and 2
3.42[a]	ATP citrate lyase
3.32[a]	Cytochrome P450, family 4, subfamily A, polypeptide 22
3.23[a]	Serine dehydratase-like
3.19[a]	Cytochrome P450, family 2, subfamily b, polypeptide 2
3.17[a]	Glutamate oxaloacetate transaminase 1
3.11[a]	Glycerol-3-phosphate acyltransferase, mitochondrial
3.08[a]	Similar to mKIAA1002 protein
3.06[a]	Sterol regulatory element binding factor 1
3.02[a]	Centaurin, γ3
Downregulated genes	
0.07[a]	Serine protease inhibitor
0.08[a]	Cysteine sulfinic acid decarboxylase
0.09[a]	Fatty acid desaturase 1
0.11[a]	Cytochrome P450, family 3, subfamily a, polypeptide 11
0.23[a]	Aldo-keto reductase family 1, member C12
0.24[a]	ATP-binding cassette, subfamily C (CFTR/MRP), member 2
0.28[a]	Cytochrome P450, family 3, subfamily a, polypeptide 18
0.28[a]	Solute carrier organic anion transporter family, member 1b2
0.28[a]	Complement component 6
0.29[a]	Hydroxysteroid (17-ß) dehydrogenase 2

[a]$P<0.05$ vs SHRSP

syndrome and that rats may be a useful tool to investigate the molecular mechanisms underlying the metabolic syndrome (11).

In this study, we identified small lipoprotein particles using HPLC. The size of this lipoprotein was similar to the sdLDL, which is one of the major features of lipid abnormality in metabolic syndrome. The existence of apolipoprotein B in this lipoprotein fraction was confirmed by Western blot analysis. From these data, a novel rat model of metabolic syndrome, SHRSP fatty, was described which carries sdLDL like particles, a major characteristic of this disease. Several studies reported differences in LDL particle size, density, and composition between patients with coronary heart disease (CHD) and healthy

controls. Prospective, case-control studies confirmed that the presence of sdLDL particles is associated with more than a 3-fold increase in CHD risk (24,25). However, the sdLDL phenotype rarely occurs as an isolated disorder. Instead, it is most frequently accompanied by hypertriglyceridemia, reduced HDL-cholesterol levels, abdominal obesity, and insulin resistance (26,27).

Therefore, the establishment of an animal model carrying sdLDL particles is an important step in examining the mechanisms which underlie the formation of these abnormal lipoproteins. Qiu *et al* reported that hepatic lipase deficient mice have sdLDLs but triglyceride enrichment was not observed in these mice (28), therefore, this is an animal model carrying sdLDLs, but this rat is a model of monogenic disease characterized by hepatic lipase deficiency. In contrast, SHRSP fatty rats have sdLDL-like particles with all of the metabolic syndrome characteristics, and is a useful tool in exploring the mechanism of the formation of lipid abnormalities in metabolic syndrome.

Cholesterol content in HDL fraction separated by gel filtration was significantly higher in SHRSP fatty compared with that in SHRSP. Plasma HDL level of SHRSP fatty is determined by the interaction of carrying genes from leptin receptor region of ZF strain and background SHRSP genes.

Fenofibrate treatment selectively decreased the small particle size of apolipoprotein B containing lipoprotein in SHRSP fatty rats. In the past, fenofibrate was reported to have an effect of increasing LDL particle size and reducing the prevalence of sdLDL in patients (29). Based on these data, the small LDL particles in SHRSP fatty rats have several similarities with human sdLDLs. Therefore, the mechanism of the formation of small particle LDLs in SHRSP fatty seems to overlap that of sdLDLs in humans.

Because of these similarities between the small LDLs in SHRSP fatty rats and human sdLDLs, we conducted an examination of the gene expression resulting in conditions that could be very informative for exploring the underlying mechanism associated with this condition. As the liver is the most important organ in the production of lipid or fatty acid-related metabolism enzymes, we examined liver gene expression in SHRSP fatty rats and control lean SHRSP rats.

Pathologic findings of the liver in the SHRSP fatty rats showed a marked accumulation of lipid droplets in hepatocytes, indicating severe fatty livers. As Adiels *et al* reported, the overproduction of VLDL is driven by the liver fat mass (20). Thus, the examination of the hepatic gene expression profile in SHRSP fatty rats is likely to be very important for understanding the mechanism of this lipid abnormality.

Seventeen genes were upregulated more than 3-fold in the liver tissue of SHRSP fatty rats. Of these genes, at least four including stearoyl coenzyme A desaturase 1 (SCD-1), fatty acid synthase (FAS), ATP citrate lyase, and sterol regulatory element binding factor 1 (SREBF1) are involved in fatty acid metabolism. In fact, SCD-1 gene expression was more than 25 times higher in the liver of SHRSP fatty rats than in control SHRSP rats. Feeding previously fasted animals a low-fat/high-carbohydrate diet caused a marked induction of enzymes involved in catalyzing fatty acid desaturation steps (30), including ATP citrate lyase (31), FAS for lipogenesis (32), and SCD-1. Specifically, SCD-1 catalyzes the introduction of a

double bond in the Δ9 position, between carbons 9 and 10, of a variety of fatty acyl CoA substrates (33), and SCD-1 is also known to play a major role in regulating the fatty acid composition of tissues (34). Ntambi *et al* reported that SCD-/- mice are resistant to diet induced obesity (35). Based on data from animal models, SCD-1 recently became a target of interest for the reversal of hepatic steatosis and insulin resistance (32). Because FAS catalyzes the last step in the fatty acid biosynthetic pathway, it is believed to be a determinant of the maximal capacity of a tissue, including the liver, to synthesize fatty acids via *de novo* lipogenesis. Furthermore, SREBF1 is known to activate the transcription of SCD-1 and FAS, as well as acyl CoA carboxylase-1 in addition to the rate-limiting enzyme in glycerolipid formation, glycerol-3-phosphate acyl-transferase. Therefore, as four of the upregulated genes in the SHRSP fatty rats, SCD-1, FAS, ATP citrate lyase and SREBF play a central role in fatty acid synthesis, upregulation of these genes resulted in increased fat content in the liver and induced overproduction of VLDL particle.

Additionally, our studies also show that some genes were significantly downregulated in SHRSP rats. For example, hepatic lipoprotein overproduction was shown as one of the features of familial combined hyperlipidemia (36), another metabolic disease producing sdLDL particles. Overproduction of these four genes may influence the production of small apolipoprotein B containing particles in SHRSP fatty rats. In contrast, fatty acid desaturase 1 (FADS1) expression was significantly decreased in SHRSP fatty rats compared to SHRSP rats. FADS1 and the human Δ5 desaturase share identical nucleotide sequences in their open reading frame with the exception of 6 alterations. Montanaro *et al* reported that SCD-1 gene expression in the liver of diabetic rats was upregulated, whereas Δ5 desaturase was not significantly modified (37). The downregulated FADS1 and other upregulated genes in our study play distinct roles in liver fatty acid metabolism. As SHRSP fatty rats showed marked accumulation of lipid droplets in hepatocytes, the genes listed in our microarray result analysis play a role in fatty liver.

In conclusion, SHRSP fatty rats demonstrate a novel model of metabolic syndrome, carrying sdLDL like lipoprotein, and thus they may be a good tool for the study of metabolic syndrome. Specifically, some of the upregulated genes in the liver of SHRSP fatty rats, SCD-1, FAS and SREBF, are believed to play a role in the metabolic abnormality of this rat model, and these genes are likely to be a therapeutic target of this metabolic syndrome.

Acknowledgements

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Original Article

Association of SLC6A9 Gene Variants with Human Essential Hypertension

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Aim: We previously identified a quantitative trait locus (QTL) on rat chromosome 5 that appeared to be primarily controlled by the sympathetic nervous system. Because sympathetic overactivity is related to hypertension, solute carrier family 6, member 9 (SLC6A9) is a candidate gene for the connection of this QTL with blood pressure regulation. In the present study, we therefore explored the role of SLC6A9 genetic variations in human essential hypertension (EH).

Methods: We evaluated three single nucleotide polymorphisms (SNPs) (rs2286245, rs3791124 and rs2486001) in 758 essential hypertension patients and 726 controls. Polymorphism-related genotypes were determined with TaqMan assays.

Results: The allelic frequency of rs2286245 (C versus T, $p = 0.032$) showed significant differences between EH and normotensive controls (NT) groups. The genotypic distribution of rs3791124 in its dominant model (AA + GA versus GG, $p = 0.027$) also showed significant differences between EH and NT groups. The genotype and allele distributions of rs2486001 did not exhibit any significant differences.

Conclusion: We found an association between SLC6A9 gene polymorphisms and essential hypertension in a Japanese population, suggesting that SLC6A9 is a susceptibility locus for essential hypertension.

Introduction

Hypertension affects 25% of most adult populations in industrialized countries[1] and is a major risk factor for stroke and coronary heart disease[2-5]. The largest group of hypertensive patients has essential

hypertension (EH), a polygenic disorder that results from the inheritance of a number of susceptibility genes. There are most likely several causal genes, which together account for 30% to 50% of the blood pressure variation found among individuals[6]. EH subjects happen to have inherited an aggregate of genes related to hypertension and/or to have been exposed to exogenous factors that predispose them to hypertension.

Using experimental crosses derived from genetically hypertensive and control rat strains, quantitative trait loci (QTL) influencing blood pressure have been mapped to several rat chromosomes[7-11]. We previously established a new approach to estimate the contribution of blood pressure regulatory systems, using a pharmacogenetic approach, and systematically analyzed the cosegregation of genetic loci for acute cardiovascular responses to drugs that influence the rennin-angiotensin system, sympathetic nervous system, or nitric oxide system in F2 populations derived from cross-breeding Prague hypertensive-hypertriglyceridemic rats with normotensive Lewis rats. In this analysis, we identified a QTL on rat chromosome 5 that correlated with basal mean arterial blood pressure. The correlation of this locus to blood pressure disappeared after pentolinium administration, thereby suggesting modulation by the sympathetic nervous system[12]. By comparative mapping, 142 annotated genes were identified in the syntenic locus of this rat QTL on human chromosome 1. Similas to most loci mapped, this locus included a large segment of the genome, and these segments clearly included several potential candidate genes.

Termination of synaptic activity is thought to occur through removal of the neurotransmitter from the synaptic cleft by ion-coupled, high-affinity neurotransmitter transport proteins located in neuronal and glial plasma membranes. Neurotransmitter transporters are membrane-bound proteins that actively transport the released neurotransmitters back into presynaptic neurons and surrounding glia, thereby terminating the activity of monoamine and amino acid neurotransmitters, and helping to replenish presynaptic pools of neurotransmitters[13, 14].

Glycine has two functions in the central nervous system. Firstly, it is an inhibitory neurotransmitter acting on strychnine-sensitive glycine receptors[15], which are located mainly in the brainstem and spinal cord. Secondly, in a broader action throughout the CNS, it regulates glutamatergic neurotransmission by acting as an obligatory coagonist of glutamate at the N-methyl-D-asparate (NMDA) receptor[16]. The actions of glycine are terminated by diffusion and/or uptake. Uptake of glycine is achieved by glycine transporters[17]. Gly-

cine transport is mediated by two sodium-dependent carriers, solute carrier family 6, member 9 (SCL6A9) and solute carrier family 6, member 5 (SLC6A5), which have distinct tissue distributions[18]. With the development of potent and specific antagonists of SLC6A9, the role of SLC6A9 in maintaining subsaturating levels of glycine at the glutamatergic synapse was established[19-21].

Therefore, the SLC6A9 gene seems to be an attractive candidate gene for sympathetic nervous system-driven blood pressure regulation in the QTL that was earlier identified on rat chromosome 5[12]. In the present study, our aims were to investigate the association between human SLC6A9 and hypertension by analyzing single-nucleotide polymorphisms (SNPs) in the human SLC6A9 gene.

Method

Case-Control Study: Collaborative Study with the Hypertension Section of the Japanese Millennium Project

The study population consisted of 758 essential hypertension (EH) patients and 726 normotensive (NT) healthy control subjects who were recruited through a subgroup collaboration with the hypertension section of the Japanese Millennium Project. Six medical institutes took part in the collaborative study and collected data on hypertensive cases and controls. Hypertensive patients were defined as having SBP 140 mmHg or DBP 90 mmHg, or were receiving chronic antihypertensive medication. To increase the statistical power of the present study, hypertensive subjects additionally had to meet the following criteria: age 60 years old or onset of hypertension at 50 years of age, a family history of hypertension, and not obese (body mass index 26 kg/m^2). NT criteria were as follows: SBP/DBP 130/85 mmHg, without a family history of hypertension, and not obese. Both groups were recruited throughout Japan, and informed consent was obtained from each individual as per the protocol approved by each institution's human studies committee.

Genotyping

Based on allelic frequency information from the web site of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nih.gov), 3 SNPs in the human SLC6A9 gene with minor allele frequencies higher than 15% were selected. SNPs with relatively high minor allele frequencies have demonstrated utility as genetic markers for genetic case-control studies. Both SNPs were confirmed using the

Table 1. Location of SNPs analyzed in the present case-control study

dbSNP rs# cluster ID	Contig position	Region	Function	dbSNP allele
rs2486001	14447905	intron 3	intron	A/G
rs3791124	14436123	intron 12	intron	A/G
rs2286245	14435122	exon 14	3'UTR	C/T

NCBI web site with accession numbers rs2286245, rs3791124 and rs2486001. We examined the association between EH and these 3 SNPs (Table 1).

Genotypes were determined using Assay-on-Demand kits (Applied Biosystems) together with Taq-Man PCR. TaqMan Universal Master Mix (PE Biosystems) was used for PCR in a 25 μL reaction volume containing 50 ng DNA, 700 nM primer, and 100 nM probe final concentrations. Thermal cycling conditions consisted of 95℃ for 10 minutes and then 40 cycles 92℃ for 15 s and 60℃ for 1 minute in a Gene-Amp 9700 system. Fluorescence levels of PCR products were measured using an ABI PRISM 7700 Sequence Detector (Applied Biosystems), which resulted in the clear identification of three genotypes for the two alleles.

Statistical Analysis

Data are shown as the mean and SD. All statistical analyses were conducted using StatView 5.0 (SAS Inc.) and Dr. SPSS Ⅱ (SPSS Inc.). Hardy-Weinberg equilibrium was assessed by two analyses. The overall distributions of the genotypes or alleles were analyzed by two analyses using 2×3 or 2×2 contingency tables between EH patients and NT controls.

Results

We previously identified a QTL on rat chromosome 5 between D5Rat147 and D5Rat108 that appeared to affect blood pressure regulation. SLC6A9 was located in the middle of the region. This rat locus was syntenic with a region on human chromosome 1. Mapping data for the human and rat locus were taken from NCBI (Fig. 1).

Basic characteristics of the patient and control groups are given in Table 2. EH patients had a significantly higher body mass index, as well as systolic and diastolic blood pressure; 499 of 758 patients with essential hypertension were treated with oral antihypertensive agents.

We performed a case-control study of the rs2286245, rs3791124 and rs2486001 SNPs using 758 EH patients and 726 NT controls (Table 3). The observed and expected genotypic frequencies of each

SNP in NT subjects were in good agreement with the predicted Hardy-Weinberg equilibrium values.

The allelic frequency of rs2286245 (C versus T, $p = 0.032$) showed significant differences between EH and NT groups. The genotypic distribution of rs3791124 in its dominant model (AA + GA versus GG, $p = 0.027$) also showed significant differences between EH and NT groups. The odds ratio of rs3791124 and rs2286245 for hypertension was estimated to be 1.32 (95%CI: 1.06 to 1.64; $p = 0.01$) and 1.26 (95%CI: 0.99 to 1.62; $p = 0.06$) after age and BMI adjustment in the dominant model.

Discussion

We performed a genetic case-control study in a Japanese population, and found that SNPs in the SLC6A9 gene were associated with EH. To our knowledge, this is the first study that relates SLC6A9 gene polymorphism to hypertension in humans.

We have established a new approach to estimate the contribution of blood pressure regulatory systems, using a pharmacogenetic approach[12]. In this analysis, the disappearance of a QTL for mean arterial pressure after blockade of the sympathetic nervous system by pentolinium reveals the contribution of the sympathetic nervous system to baseline mean arterial pressure. Using this approach, we determined that the baseline mean arterial pressure of the hypertensive rat strain was controlled by the QTL on rat chromosome 5 through the sympathetic nervous system.

Dense gene maps have been established for the rat by the Rat Genome Database and NCBI. The human genome project has enabled construction of a human genome map that is currently available from NCBI. These maps can be used to project the results of quantitative genetic analysis of rat chromosomes onto the human genome. We have previously reported a strategy to extrapolate data from rat quantitative trait genetics onto the human genome, using a comparative mapping approach[22]. In the current study, we applied our comparative approach to our previously identified QTL for baseline mean arterial pressure on rat chromosome 5, to which contribution of the sympathetic nervous system was suggested, and selected
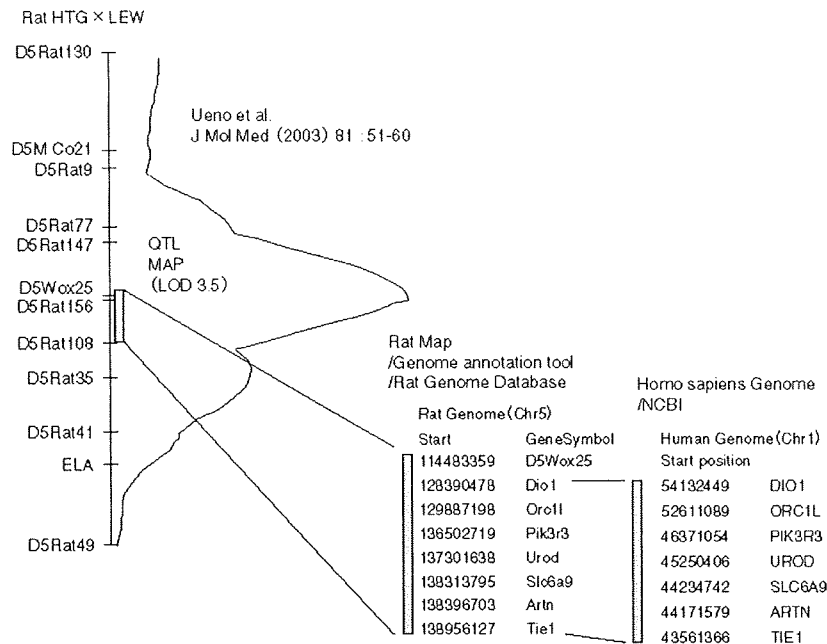
Fig. 1. Comparative map of rat chromosome 5 and human chromosome 1.

Comparative mapping was performed using HTG×LEW map[22], Rat Genome Map and Homo Sapiens Genome Map of National Center for Biotechnology Information (NCBI) (http://www.ncbi.nih.gov).

Dio1, deiodinase, iodothyronine, type I; Orc1l, origin recognition complex, subunit 1-like; Pik3r3, phosphatidylinositol 3 kinase, regulatory subunit, polypeptide 3; Urod, uroporphyrinogen decarboxylase; solute carrier family 6, member 9; Artn, artemin; Tie1, tyrosine kinase with immunoglobulin-like and EGF-like domains 1

Table 2. Clinical characteristics of NT and EH

	EH	NT
Number of subjects	758	726
Male (%)	564 (74.4)	550 (75.8)
Age	59.0 ± 11.0*	62.8 ± 9.4
Body mass index (kg/m^2)	23.6 ± 3.0*	22.7 ± 2.9
Systolic blood pressure (mmHg)	163.5 ± 24.6*	115.9 ± 12.0
Diastolic blood pressure (mmHg)	100.3 ± 15.7*	72.0 ± 7.6
Antihypertensive medication, n (%)	499 (65.8)	

*: $p < 0.05$ by Mann-Whitney's U-test.
NT: normotensive control, EH: essential hypertension

SLC6A9 as a possible candidate gene for blood pressure regulation.

Although sympathetic nervous activation in essential hypertension has been well documented, with analysis of the regional sympathetic nervous system function demonstrating activation of sympathetic nervous outflows to the heart, kidneys and skeletal muscle vasculature[23], the exact pathophysiology of sympathetic nervous dysfunction remains to be delineated. Termination of synaptic activity is thought to occur through neurotransmitter removal from the synaptic cleft by ion-coupled, high-affinity neurotransmitter transporter proteins. SLC6A9 have been suggested to play an important regulatory role at the glycine receptor containing synapses by clearing glycine from the synaptic cleft, and at synapses containing NMDA receptors by maintaining the extracellular glycine level below saturating concentrations at the glycine site on NMDA receptors[24-27]. As there is increasing evidence that essential hypertension, at least in its early stages, is accompanied by sympathetic hyperactivation, the contribution of the central nervous system to essential hypertension has been reported. Concerning this evidence, SLC6A9 seems to be an attractive candidate gene for essential hypertension, and this gene plays an important role in maintaining sympathetic activity in the central nervous system.

Recently, the association of SLC6A9 polymor-