Association of polymorphisms of *THBS2* and *HSPA8* with hypertension in Japanese individuals with chronic kidney disease

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Abstract. The purpose of the present study was to identify gene polymorphisms that confer susceptibility to hypertension in individuals with chronic kidney disease (CKD), thereby contributing to the prediction of genetic risk for this condition. The study population comprised 1824 Japanese individuals with CKD [estimated glomerular filtration rate (eGFR) <60 ml min^{-1} 1.73 m^{-2}], including 1257 subjects with hypertension and 567 controls. The genotypes for 50 polymorphisms of 46 candidate genes were determined using a method that combines the polymerase chain reaction and sequence-specific oligonucleotide probes with suspension array technology. An initial screen of allele frequencies by the χ^2 test revealed that two polymorphisms were significantly (false discovery rate <0.05) associated with the prevalence of hypertension in individuals with CKD. Subsequent multivariable logistic regression analysis with adjustment for age, gender and the prevalence of diabetes mellitus revealed that these two polymorphisms, 3949T→G (3'-UTR) of the thrombospondin 2 gene (*THBS2*; odds ratio in recessive model, 8.31) and -110A→C of the heat shock 70-kDa protein 8 gene (*HSPA8*; odds ratio in recessive model, 0.72) were significantly (P<0.05) associated with the prevalence of hypertension. The variant *G* allele of *THBS2* was a risk factor for hypertension, whereas the variant *C* allele of *HSPA8* was protective against this condition. A stepwise forward selection procedure also demonstrated that the *THBS2* and *HSPA8* genotypes were significant (P<0.05) and independent determinants of hypertension. Determination of genotypes for these polymorphisms may prove informative for the prediction of genetic risk for hypertension in Japanese individuals with CKD. Validation of these findings will require additional studies with independent subject panels.

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Introduction

Individuals with chronic kidney disease (CKD) frequently suffer from hypertension, which is sometimes intractable, making it difficult to achieve the target blood pressure (1-3). Hypertension in patients with CKD is an independent predictor of both the progression of CKD and the development of cardiovascular disease (3-7). Strict control of blood pressure is thus highly desirable in such patients and is thought to delay the progression of renal injury (8). The identification of genetic markers for hypertension and subsequent personalized intervention at an early stage of CKD may prove effective in reducing the prevalence of cardiovascular disease. However, genetic factors underlying predisposition to hypertension in individuals with CKD have remained largely uncharacterized. Furthermore, given ethnic differences in lifestyle and environmental factors, as well as in genetic background and renal function, it is important to examine genetic variants related to hypertension in individuals with CKD in each ethnic group.

In the present study, we performed an association study for 50 candidate gene polymorphisms and hypertension in 1824 Japanese individuals with CKD. The purpose of the study was to identify genetic variants that confer susceptibility to hypertension in individuals with CKD, thereby providing a basis for the personalized prevention of this condition.

Table I. Primers, probes and additional polymerase chain reaction conditions for the genotyping of polymorphisms associated with hypertension in individuals with chronic kidney disease.

Gene	Polymorphism	Primers		Probes		Annealing temp. (˚C)	Cycles
THBS2	3949T→G	Sense	AACCCAAGTGCCTTCAGAGGAT	Probe 1	GATGTTCATCTCTGAGTTCCA	60	50
	(3'-UTR)	Antisense	CTCCACATAAAGTCTCATATATCAC	Probe 2	GATGTTCATCTCTGCGTTCCA		
HSPA8	-110A→C	Sense	CAGGGGGCGGCATTCTGGC	Probe 1	GAATATTCCAGGGTTTTCGCCT	60	50
		Antisense	CTTCTGGGCCAATCACCGAG	Probe 2	ACGGGAGGCGAAACCCCTG		

Materials and methods

Study population. The study population comprised 1824 unrelated Japanese individuals (1120 males, 704 females) with CKD who visited the outpatient clinics of or were admitted to one of the participating hospitals: Gifu Prefectural General Medical Center and Gifu Prefectural Tajimi Hospital (Gifu Prefecture, Japan), and Hirosaki University Hospital, Reimeikyo Rehabilitation Hospital and Hirosaki Stroke Center (Aomori Prefecture, Japan) between October 2002 and March 2008 due to various symptoms or for an annual health checkup. Also included were individuals recruited to a population-based prospective cohort study of aging and age-related diseases in Gunma Prefecture and Tokyo, Japan.

Estimated glomerular filtration rate (eGFR) was calculated using the simplified prediction equation proposed by the Japanese Society of Nephrology and based on that described in the Modification of Diet in Renal Disease (MDRD) Study: eGFR (ml min^{-1} 1.73 m^{-2}) = 194 x [age (years)]$^{-0.287}$ x [serum creatinine (mg/dl)]$^{-1.094}$ [x 0.739 if female]. The National Kidney Foundation-Kidney Disease Outcomes Quality Initiative guidelines recommend a diagnosis of CKD if eGFR is <60 ml min^{-1} 1.73 m^{-2} (9). Non-linear relations between GFR and the risk of adverse outcomes, such as death, cardiovascular events and hospitalization, have been demonstrated, with increased risk being associated with an eGFR <60 ml min^{-1} 1.73 m^{-2} and markedly increased risk with values falling below 45 ml min^{-1} 1.73 m^{-2} (3). Therefore, the criterion of an eGFR <60 ml min^{-1} 1.73 m^{-2} was adopted for the diagnosis of CKD in the present study.

The 1257 subjects (789 males, 468 females) with hypertension had a systolic blood pressure ≥160 mmHg and/or a diastolic blood pressure ≥95 mmHg prior to medical treatment. Individuals with valvular heart disease, congenital malformations of the heart or vessels, or renal or endocrinologic diseases that cause secondary hypertension were excluded from the study. The control subjects comprised 567 individuals (331 males, 236 females) who visited the out-patient clinics of the participating hospitals for an annual health checkup or were community-dwelling individuals in the prospective cohort study with normal blood pressure (systolic blood pressure <140 mmHg and diastolic blood pressure <90 mmHg) and no history of hypertension or of taking antihypertensive medication. The hypertensive and control individuals had or did not have diabetes mellitus, hypercholesterolemia or obesity.

Blood pressure was measured at least twice, with subjects resting in a sitting position for >5 min prior to measurement. Measurements were taken by a skilled physician according to the guidelines of the American Heart Association (10). The study protocol complied with the Declaration of Helsinki and was approved by the Committees on the Ethics of Human Research of Mie University Graduate School of Medicine, Hirosaki University Graduate School of Medicine, Gifu International Institute of Biotechnology, Tokyo Metropolitan Institute of Gerontology, and participating hospitals. Written informed consent was obtained from each participant.

Selection and genotyping of polymorphisms. With the use of public databases, including PubMed (NCBI) and Online Mendelian Inheritance in Man (NCBI), we selected 46 candidate genes that have been characterized and proposed to be associated with hypertension. On the basis of published studies or by searching PubMed and single nucleotide polymorphism (SNP) databases [dbSNP (NCBI) and Japanese SNP (JSNP)], we further selected 50 polymorphisms of these genes, most of which were located in the promoter region or exons, that might be expected to result in changes in the function or expression of the encoded protein (data not shown). Wild-type and variant alleles of the polymorphisms were determined from the original sources.

Venous blood (7 ml) was collected in tubes containing 50 mmol/l EDTA (disodium salt), and genomic DNA was isolated using a kit (Genomix; Talent, Trieste, Italy). Genotypes of the 50 polymorphisms were determined at G&G Science (Fukushima, Japan) by a method that combines the polymerase chain reaction and sequence-specific oligonucleotide probes with suspension array technology (Luminex, Austin, TX). Primers, probes and other conditions for the genotyping of polymorphisms significantly associated with CKD are shown in Table I. Detailed genotyping methodology was as previously described (11).

Statistical analysis. Quantitative data were compared between subjects with hypertension and the controls by the unpaired Student's t-test. Categorical data were compared by the χ^2 test. Allele frequencies were estimated by the gene counting method, and the χ^2 test was used to identify departure from Hardy-Weinberg equilibrium. In an initial screening, allele frequencies (2x2) of each polymorphism from subjects with hypertension and controls were compared using the χ^2 test. Given the multiple comparisons of genotypes, the false

Table II. Characteristics of subjects with hypertension and controls among individuals with chronic kidney disease.

Characteristic	Hypertension	Controls	P-value
No. of subjects	1257	567	
Age (years)	71.0±8.7	70.6±9.3	0.4219
Gender (male/female, %)	62.8/37.2	58.4/41.6	0.0753
Body mass index (kg/m^2)	23.5±3.4	23.3±3.3	0.1572
Current or former smoker (%)	20.5	23.6	0.1414
Systolic blood pressure (mmHg)	154±26	128±17	<0.0001
Diastolic blood pressure (mmHg)	82±15	73±12	<0.0001
Hypercholesterolemia (%)	31.0	28.0	0.2088
Serum total cholesterol (mmol/l)	5.22±1.02	5.23±1.00	0.8676
Serum triglyceride (mmol/l)	1.70±1.09	1.57±0.94	0.0194
Serum HDL-cholesterol (mmol/l)	1.32±0.42	1.4 ±0.40	0.0001
Diabetes mellitus (%)	41.2	21.0	<0.0001
Fasting plasma glucose (mmol/l)	7.10±3.10	6.69±2.91	0.0247
Glycosylated hemoglobin (%)	6.01±1.52	5.65±1.37	0.0008
Serum creatinine (μmol/l)	119.8±135.8	91.7±26.2	<0.0001
eGFR (ml min^{-1} 1.73 m^{-2})	47.4±11.8	51.0±8.0	<0.0001
End-stage renal disease (%)	3.6	0.4	<0.0001

Quantitative data are the means ± SD. Smoker, smoking ≥10 cigarettes daily. Hypercholesterolemia, serum total cholesterol ≥5.72 mmol/l (220 mg/dl) or taking lipid-lowering medication. Diabetes mellitus, fasting plasma glucose ≥6.93 mmol/l (126 mg/dl), glycosylated hemoglobin (hemoglobin A1c) content ≥6.5%, or taking antidiabetes medication.

discovery rate (FDR) was calculated (12) from the distribution of P-values for allele frequencies of the 50 polymorphisms. Polymorphisms with an FDR <0.05 were further examined by multivariable logistic regression analysis with adjustment for covariates. Multivariate logistic regression analysis was thus performed with hypertension as a dependent variable and with independent variables including age, gender (0, female; 1, male), the prevalence of diabetes mellitus (0, no history of the condition; 1, positive history), and the genotype of each polymorphism. The P-value, odds ratio and 95% confidence interval were calculated. Each genotype was assessed according to dominant (0, wild-type homozygote; 1, heterozygote = variant homozygote), recessive (0, wild-type homozygote = heterozygote; 1, variant homozygote), and additive [(0,0) wild-type homozygote; (1,0), heterozygote; (0,1), variant homozygote] genetic models. Additive models included the additive 1 model (heterozygotes versus wild-type homozygotes) and the additive 2 model (variant homozygotes versus wild-type homozygotes), which were analyzed simultaneously using a single statistical model. We also performed a stepwise forward selection procedure to examine the effects of genotypes as well as of other covariates on hypertension. The P-value levels for inclusion in and exclusion from the model were 0.25 and 0.1, respectively. In the stepwise forward selection procedure, each genotype was examined according to a dominant or recessive model on the basis of statistical significance in the multivariable logistic regression analysis. With the exception of the initial screening by the χ^2 test (FDR <0.05), a P-value <0.05 was considered statistically significant. Statistical significance was examined by two-sided tests performed with JMP version 5.1 software and JMP Genomics version 3.2 software (SAS Institute, Cary, NC).

Results

The characteristics of the 1824 study subjects are shown in Table II. In the subjects with hypertension, the prevalence of diabetes mellitus and end-stage renal disease, the serum concentrations of triglyceride and creatinine, the fasting plasma glucose concentration, the blood glycosylated hemoglobin content, as well as systolic and diastolic blood pressure were greater than in the controls, whereas the serum concentration of high density lipoprotein (HDL)-cholesterol and eGFR were lower than in the controls.

Evaluation of allele frequencies by the χ^2 test revealed that 14 polymorphisms were related (P-value for allele frequency <0.05) to the prevalence of hypertension. Of these, 3949T→G (3'-UTR) of the thrombospondin 2 gene (*THBS2*) and -110A→C of the heat shock 70-kDa protein 8 gene (*HSPA8*) were significantly associated with the prevalence of hypertension on the basis of an FDR for allele frequency <0.05 (Table III). The genotype distributions for all 14 polymorphisms related to hypertension are also shown in Table III. Those for the two polymorphisms significantly associated with this condition were in Hardy-Weinberg equilibrium in both hypertensive and control subjects (Table IV).

Multivariate logistic regression analysis with adjustment for age, gender and the prevalence of diabetes mellitus revealed that the 3949T→G (3'-UTR) polymorphism (rs8089) of *THBS2* (dominant, recessive and additive 2 models) and the -110A→C polymorphism (rs1008438) of *HSPA8* (dominant, recessive and additive 2 models) were significantly (P<0.05) associated with hypertension (Table V). The variant *G* allele of *THBS2* was a risk factor for hypertension, whereas the variant *C* allele of *HSPA8* was protective against this condition.

Table III. Genotype distributions of polymorphisms related (P-value for allele frequency <0.05) to hypertension among individuals with chronic kidney disease as determined by the χ^2 test.

Gene symbol	Polymorphism	dbSNP	Hypertension	Controls	P-value	FDR
THBS2	3949T→G (3'-UTR)	rs8089			0.0012	0.048
	TT		1011 (80.4)	487 (85.9)		
	TG		227 (18.1)	79 (13.9)		
	GG		19 (1.5)	1 (0.2)		
HSPA8	-110A→C	rs1008438			0.0028	0.048
	AA		432 (34.4)	165 (29.1)		
	AC		584 (46.5)	263 (46.4)		
	CC		241 (19.2)	139 (24.5)		
GCK	-30G→A	rs1799884			0.0052	0.065
	GG		877 (69.8)	366 (64.6)		
	GA		348 (27.7)	173 (30.5)		
	AA		32 (2.6)	28 (4.9)		
APOC3	-482C→T	rs2854117			0.0102	0.102
	CC		337 (26.8)	192 (33.9)		
	CT		634 (50.4)	258 (45.5)		
	TT		286 (22.8)	117 (20.6)		
GHSR	A→G	rs509035			0.0127	0.106
	AA		216 (17.2)	87 (15.4)		
	AG		609 (48.5)	246 (43.5)		
	GG		430 (34.3)	233 (41.2)		
IL1B	-511C→T	rs16944			0.0152	0.108
	CC		362 (28.8)	196 (34.6)		
	CT		606 (48.2)	257 (45.3)		
	TT		289 (23.0)	114 (20.1)		
MMP3	A→G (Lys45Glu)	rs679620			0.0259	0.138
	AA		143 (11.4)	75 (13.2)		
	AG		524 (41.7)	258 (45.5)		
	GG		590 (46.9)	234 (41.3)		
FBN1	1875T→C	rs25458			0.0316	0.138
	TT		388 (30.9)	194 (34.2)		
	TC		609 (48.5)	280 (49.4)		
	CC		260 (20.7)	93 (16.4)		
ALOX5AP	162A→C	rs4769055			0.0321	0.138
	AA		321 (25.6)	124 (21.9)		
	AC		634 (50.5)	285 (50.3)		
	CC		300 (23.9)	158 (27.9)		
PTGIS	1117C→A	rs5629			0.0350	0.138
	CC		764 (60.8)	313 (55.2)		
	CA		436 (34.7)	224 (39.5)		
	AA		57 (4.5)	30 (5.3)		
GCLC	-129C→T	rs17883901			0.0391	0.138
	CC		957 (76.1)	406 (71.6)		
	CT		284 (22.6)	151 (26.6)		
	TT		16 (1.3)	10 (1.8)		

Table III. Continued.

Gene symbol	Polymorphism	dbSNP	Hypertension	Controls	P-value	FDR
OR13G1	A→G (Ile132Val)	rs1151640			0.0420	0.138
	AA		174 (13.9)	67 (11.8)		
	AG		592 (47.2)	251 (44.3)		
	GG		489 (39.0)	249 (43.9)		
MMP1	-1607/1G→2G	rs1799750			0.0434	0.138
	1G1G		135 (10.7)	80 (14.1)		
	1G2G		566 (45.0)	256 (45.2)		
	2G2G		556 (44.2)	231 (40.7)		
F7	11496G→A (Arg353Gln)	rs6046			0.0466	0.138
	GG		1119 (89.0)	487 (85.9)		
	GA		133 (10.6)	76 (13.4)		
	AA		5 (0.4)	4 (0.7)		

Numbers in parentheses are percentages.

Table IV. Hardy-Weinberg P-values for subjects with hypertension and controls.

Gene	Polymorphism	Hypertension	Controls
THBS2	3949T→G (3'-UTR)	0.1322	0.2307
HSPA8	-110A→C	0.0833	0.0939

Finally, we performed a stepwise forward selection procedure to examine the effects of the genotypes of the polymorphisms associated with hypertension by multivariate logistic regression analysis, as well as the effects of age, gender and the prevalence of diabetes mellitus on hypertension (Table VI). Diabetes mellitus, the *HSPA8* genotype (recessive model) and the *THBS2* genotype (dominant model), in descending order of statistical significance, were significant ($P<0.05$) and independent determinants of hypertension in individuals with CKD.

Discussion

We examined the possible relations of 50 polymorphisms in 46 candidate genes to the prevalence of hypertension in 1824 Japanese individuals with CKD. Our association study revealed that the 3949T→G (3'-UTR) polymorphism of *THBS2* and the -110A→C polymorphism of *HSPA8* were significantly associated with the prevalence of hypertension in such individuals.

Thrombospondin 2 (THBS2), a member of the thrombospondin family, is an extracellular matrix glycoprotein (13) that plays an important role in cell adhesion, migration and proliferation, as well as in angiogenesis. It is also implicated in atherosclerosis and thrombosis due to its function in the regulation of matrix metallopeptidase 2 (MMP2), a potential determinant of plaque vulnerability (14,15). THBS2-deficient fibroblasts were shown to produce twice as much MMP2 as wild-type cells (16). However, THBS2 has not been detected in human plasma (17). The 3949T→G (3'-UTR) polymorphism of *THBS2* was previously shown to be associated with myo-

Table V. Multivariable logistic regression analysis of polymorphisms associated (FDR <0.5) with hypertension by the χ^2 test for individuals with chronic kidney disease.

Gene symbol	Polymorphism	Dominant		Recessive		Additive 1		Additive 2	
		P-value	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)
THBS2	3949T→G (3'-UTR)	0.0154	1.41 (1.07-1.88)	0.0400	8.31 (1.70-15.3)	0.0543		0.0361	8.68 (1.77-15.71)
HSPA8	-110A→C	0.0406	0.79 (0.64-0.99)	0.0093	0.72 (0.57-0.93)	0.2251		0.0044	0.66 (0.50-0.88)

OR, odds ratio; CI, confidence interval. Multivariable logistic regression analysis was performed with adjustment for age, gender and the prevalence of diabetes mellitus.

Table VI. Effects of genotypes and other characteristics on hypertension in individuals with chronic kidney disease determined by a stepwise forward selection procedure (P<0.05).

Variable	P-value	R^2
Diabetes mellitus	<0.0001	0.0325
HSPA8 (*CC* versus *AA* + *AC*)	0.0146	0.0027
THBS2 (*TG* + *GG* versus *TT*)	0.0164	0.0025

R^2, contribution rate.

cardial infarction, with the *G* allele being protective against this condition (17-20). However, this finding has not been consistently replicated (21). Although the genetic variation of *THBS2* has been extensively studied in individuals with various diseases, as far as we are aware it has not previously been associated with hypertension. We have now demonstrated that the 3949T→G (3'-UTR) polymorphism of *THBS2* is significantly associated with the prevalence of hypertension in individuals with CKD, with the *G* allele representing a risk factor for this condition. The risk allele thus differed between the previous studies of myocardial infarction (17-20) and the present study. Although the reason for this discrepancy is unclear, it is possible that this polymorphism is in linkage disequilibrium with other polymorphisms responsible for the development of hypertension in individuals with CKD.

The expression of heat shock proteins in cells is induced by a wide variety of mechanical or other environmental stresses, with these proteins playing important roles as molecular chaperones (22,23). They are present in the circulation of healthy individuals as well as in patients with various diseases (24). Heat shock 70-kDa protein 8 (HSPA8) is a key member of this protein family that also functions as a cytokine to stimulate a pro-inflammatory response in human monocytes (25). Its expression was induced in atherosclerotic plaques and appeared to protect arterial smooth muscle cells against plaque toxicity in an experimental model (26). The plasma level of HSPA8 has been shown to be increased in association with various diseases (27,28). Furthermore, vascular expression of HSPA8 was found to be up-regulated in individuals with atherosclerotic diseases, including hypertension and myocardial infarction, possibly as a cytoprotective response to endothelial injury (29,30). The -110A→C polymorphism of *HSPA8* has been shown to influence gene function (31), while the variant *C* allele of this polymorphism manifested lower transcriptional activity than the *A* allele (32). We now demonstrated that the -110A→C polymorphism of *HSPA8* is significantly associated with hypertension in individuals with CKD, with the *C* allele being protective against this condition. The mechanism by which a reduced transcriptional activity of *HSPA8* might protect against hypertension in individuals with CKD remains unclear.

The present study has several limitations: (i) we used eGFR rather than directly measuring GFR to define CKD; (ii) we were unable to obtain information regarding underlying renal disease in the subjects with CKD (such information can be obtained by detailed clinical examination, including renal biopsy, but these diagnostic procedures are not considered feasible in a study whose subjects are recruited from the general population); (iii) it is possible that one or more of the polymorphisms associated with hypertension in the present study are in linkage disequilibrium with other polymorphisms in the same gene or in nearby genes that are actually responsible for the development of this condition; and (iv) the functional relevance of the association of the identified polymorphisms with hypertension was not examined.

In conclusion, our present results suggest that *THBS2* and *HSPA8* are susceptibility loci for hypertension in Japanese individuals with CKD. The determination of genotypes for the polymorphisms of these genes may prove informative for the assessment of the genetic risk of hypertension in individuals with CKD. Validation of our findings will require their replication with independent subject panels.

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