Original Article

Haplotype-Based Case-Control Study of the Association between the Guanylate Cyclase Activator 2B (GUCA2B, Uroguanylin) Gene and Essential Hypertension

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Uroguanylin (gene name: guanylate cyclase activator 2B, GUCA2B) is a peptide regulator of intestinal salt and water transport. It has been reported that the uroguanylin knockout mouse exhibits elevated blood pressure. Therefore, the GUCA2B gene is thought to be a susceptibility gene for essential hypertension (EH). Despite extensive studies, however, the relationship between the GUCA2B gene and EH has not yet been defined. The aim of this study was to assess the association between the human GUCA2B gene and EH. Using four single nucleotide polymorphisms (SNPs), we conducted a genetic association study in 281 EH patients and 279 age-matched normotensive (NT1) individuals. To derive more reliable data, we performed a duplicate case-control study in which we recruited another normotensive group (NT2). There was no significant difference in the overall distribution of alleles for any of the SNPs between the EH and NT1 groups, or between the EH and NT2 groups. Therefore, these four SNPs cannot be the genetic markers for EH. The occurrences of the C-A haplotype (rs883062-rs1047047) and the C-A-G haplotype (rs883062-rs1047047-rs2297566) were significantly higher in the EH group than in the NT1 group ($p<0.0001$) or the NT2 group ($p<0.0001$). These results suggest that the C-A haplotype and the C-A-G haplotype of the GUCA2B gene are the genetic markers for EH, and that GUCA2B or a neighboring gene might be a susceptibility gene for EH. (*Hypertens Res* 2007; 30: 789–796)

***Key Words*: haplotypes, guanylate cyclase activator 2B, single nucleotide polymorphism, association study, essential hypertension**

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Introduction

While uroguanylin is expressed primarily in the mammalian intestine, it is also found in the kidney. It was identified because of its homology to the bacterial heat-stable enterotoxin (ST) and its ability to bind to the ST receptor, guanylate cyclase-C (GC-C) (*1*, *2*). ST is a small peptide that is produced by enterotoxigenic bacterial strains and that binds and activates the receptor GC-C. GC-C activation by ST during bacterial infection increases the intracellular levels of cGMP in enterocytes, causing a secretory diarrhea. GC-C is normally regulated by the endogenous peptide ligand, uroguanylin (*3*). Uroguanylin has local intestinal (paracrine) and endocrine functions, forming a potential enteric-renal link that is involved in coordinating salt ingestion *via* natriuresis (*4–6*). Previous studies on uroguanylin peptide suggest that it may function as an endocrine intestinal natriuretic hormone, as it has been found to circulate in the bloodstream (*7*, *8*). In addition, high-salt intake can cause an increase in uroguanylin mRNA (*9*, *10*), as well as an increase in the urinary excretion of uroguanylin (*11*). It has also been noted that there is an increase in uroguanylin levels in the circulation of patients with renal disease and congestive heart failure (*12*, *13*). Previous studies using mice that had a disruption of the genes responsible for the generation of uroguanylin have also provided information on the function of these genes. Blood pressure levels for mice with the targeted homozygous uroguanylin gene (−/−) were found to be significantly higher than those seen in the heterozygous (+/−) and wild types (+/+). These results suggest that uroguanylin is a very important factor in the physiological regulation of blood pressure (*1*).

The human GUCA2B gene that encodes uroguanylin is located on chromosome 1p33-p34 (*14*). The gene consists of three exons and two introns that are found within an overall length of 2.5 kb (*15*, *16*), and has several single nucleotide polymorphisms (SNPs). Although the GUCA2B gene is thought to be a susceptibility gene for hypertension, there have been no studies that have examined the association between the GUCA2B gene and essential hypertension (EH).

High blood pressure or hypertension affects 25% of most adult populations and is an important risk factor for death from stroke, myocardial infarction and congestive heart failure. Most hypertensive cases are classified as being primary and are referred to as EH. EH is thought to be a multifactorial disease (*17*).

The aim of this study was to assess the association between the human GUCA2B gene and EH using SNPs.

Methods

Subjects

This study included a group of 281 patients that were diagnosed with EH. A positive diagnosis required the patient to have a seated systolic blood pressure (SBP) above 160 mmHg and/or diastolic blood pressure (DBP) above 100 mmHg on three occasions within 2 months after their first medical examination. None of the patients were using antihypertensive medication and subjects diagnosed with secondary hypertension were excluded. We also included 279 normotensive (NT1) healthy individuals as controls. None of the NT participants had a family history of hypertension, and all had SBP and DBP below 130 and 85 mmHg, respectively. To derive more reliable data, we performed a duplicate case-control study in which we recruited another normotensive group (NT2). The NT2 subjects consisted of 285 essentially healthy elderly Japanese (mean age, 77.8±4.2 years). They were members of a group called the New Elder Citizen Movement in Japan, for which the physical and psychosocial characteristics have been previously described (*18*). The plasma high-density lipoprotein (HDL) cholesterol concentration and serum uric acid concentration data were not collected. A family history of hypertension was defined as a prior diagnosis of hypertension in grandparents, uncles, aunts, parents or siblings. Both groups were recruited from the northern area of Tokyo, and informed consent was obtained from each individual as per the protocol approved by the Human Studies Committee of Nihon University (*19*, *20*).

Biochemical Analysis

The methodology employed by the Clinical Laboratory Department of Nihon University Hospital was used to measure all plasma total cholesterol and HDL cholesterol concentrations, and serum creatinine and uric acid concentrations (*21*).

Genotyping

We selected four SNPs in the introns of the human GUCA2B gene as markers for the genetic association experiment (Fig. 1). The minor allele frequencies for each of the SNPs among the Japanese subjects were >10%, which indicates that they all should be effective genetic markers. All SNPs were confirmed using the database for SNPs (dbSNP) on the NCBI website and the Applied Biosystems-Celera Discovery System. The accession numbers were as follows: rs883062 (C__1595618_10), rs1047047 (C__11291674_10), rs2297566 (C__1595598_10), and rs885846 (C__8909905_10) (Fig. 1). rs1047047 was located in exon 1 and exhibited no amino acid changes, thereby resulting in a silent mutation. rs883062 (C__1595618_10) was located upstream of exon 1. The other two SNPs were located in the intron.

Genotypes were determined using Assays-on-Demand kits (Applied Biosystems, Branchburg, USA) together with TaqMan® PCR. When allele-specific fluorogenic probes hybridize to the template during the polymerase chain reaction (PCR), the 5′ nuclease activity of the Taq polymerase can dis-

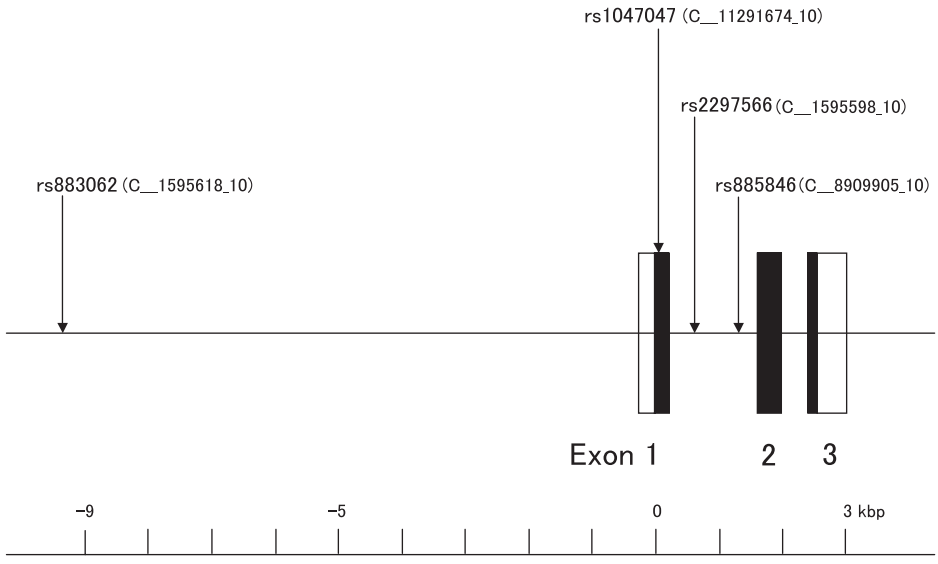

Fig. 1. *Organization of the human GUCA2B gene and location of the SNPs used for the association study. Closed black boxes indicate exons (coding region), closed white boxes indicate exons (non-coding region), and lines indicate introns.*

criminate alleles. Cleavage results in increased emission of a reporter dye that otherwise is quenched by the dye TAMRA. Each 5′ nuclease assay requires two unlabeled PCR primers and two allele-specific probes. Each probe was labeled with a reporter dye (VIC and FAM) at the 5′ end and TAMRA at the 3′ end. Amplification by PCR was carried out using TaqMan Universal Master Mix (Applied Biosystems) in a 5 μL reaction volume with final total concentrations of 2 ng DNA, 900 nmol/L primer, and 200 nmol/L probe. Thermal cycling conditions consisted of 95°C for 10 min, and then 40 cycles of 92°C for 15 s and 60°C for 1 min in a GeneAmp 9700 system.

All 96-well plates contained 80 samples of unknown genotype, eight known allele 1 homozygotes, eight known allele 2 homozygotes, and eight reactions with reagents but no DNA. The homozygote and control samples without DNA were required for the SDS 7700 signal processing that is outlined in the TaqMan Allelic Discrimination Guide (Applied Biosystems). Direct sequencing, single-stand conformation polymorphism (SSCP), or denaturing high pressure liquid chromatography was used to confirm control sample genotypes. PCR plates were read on the ABI 7700 instrument using the SDS version 1.9 software package in the end-point analysis mode (Applied Biosystems). Genotypes were visually determined by comparison with the dye-component fluorescent emission data shown in the *X-Y* scatter-plot of the SDS software. Genotypes were also automatically determined by the signal processing algorithms in the software. The results of both scoring methods were saved to two output files for later comparison.

Haplotype-Based Case-Control Study and Linkage Disequilibrium Analysis

We performed a haplotype-based case-control study using the four SNPs. Based on the genotype data of the four genetic variations, the frequency of each haplotype was estimated using the expectation/maximization (EM) algorithm (*22*, *23*). In order to determine haplotype and linkage disequilibrium (LD), SNPAlyze version 3.2.3 was used (Dynacom Co., Ltd., Yokohama, Japan), which is available from the Dynacom website at http://www.dynacom.co.jp/products/package/snpalyze/index.html. Haplotypes with a frequency of <0.02 were excluded from the analysis.

Diplotype frequency was also estimated by SNPAlyze version 3.2.3. Clinical parameters were compared between the patients with and without one haplotype by using the diplotype data.

Statistical Analysis

Data are shown as the mean±SD. Differences between the EH and NT groups were assessed by analysis of variance (ANOVA) followed by a Fisher's protected least significant difference (PLSD) test.

Hardy-Weinberg equilibrium was assessed by a χ^2 analysis. When the sizes of the expected values were small (below 2.0), the genotypes were combined (*24*). The overall distribution of the SNP alleles was analyzed by 2×2 contingency tables, and the distribution of the SNP genotypes between the EH patients and NT controls was tested using a two-sided Fisher exact test and multiple logistic regression analysis. Statistical significance was established at $p<0.05$.

Table 1. Characteristics of Study Participants

	Total					Men				
	NT1	NT2	EH	*p* value *vs*. NT1	*p* value *vs*. NT2	NT1	NT2	EH	*p* value *vs*. NT1	*p* value *vs*. NT2
Number of subjects	279	285	281			180	138	185		
Age (years)	51.9±9.6	77.8±4.2	50.6±5.9	0.055	<0.001	51.5±6.3	78.0±4.6	50.4±6.3	0.104	<0.001
BMI (kg/m^2)	22.7±3.2	22.6±2.9	24.6±3.8	<0.001	<0.001	22.9±3.2	22.8±2.7	24.8±3.6	<0.001	<0.001
SBP (mmHg)	112.6±10.9	135.1±16.5	173.4±19.7	<0.001	<0.001	113±10.3	135.1±16.5	171.2±18.3	<0.001	<0.001
DBP (mmHg)	69.6±8.3	78.1±10.7	105.4±13.4	<0.001	<0.001	70.3±7.8	78.3±9.8	105.5±13.4	<0.001	<0.001
Pulse (beats/min)	74.2±14.6	69.9±10.9	77.7±15.3	0.019	<0.001	73.4±15.8	68.9±11.9	77.6±16.0	0.027	<0.001
Creatinine (mg/dL)	0.8±0.2	0.8±0.2	0.9±0.2	0.303	0.696	0.9±0.2	0.9±0.2	0.9±0.2	0.102	0.902
Total cholesterol (mg/dL)	199.1±46.0	218.4±44.0	210.8±41.7	0.002	0.038	193.9±45.5	205.3±32.4	205.3±43.0	0.018	0.990
HDL cholesterol (mg/dL)	56.7±17.2		56.5±17.4	0.891		54.8±16.0		53.1±16.7	0.377	
Uric acid (mg/dL)	5.6±4.2		5.7±1.7	0.839		5.83±1.4		6.2±1.5	0.019	
Alcohol consumption (%)	38.7	37.0	63.0	0.070	<0.001	48.3	44.2	77.3	0.030	0.006
Smoking (%)	25.8	26.7	51.2	0.003	<0.001	33.3	41.5	62.2	0.018	<0.001

	Women				
	NT1	NT2	EH	*p* value *vs*. NT1	*p* value *vs*. NT2
Number of subjects	99	147	96		
Age (years)	53.8±1.5	77.7±3.8	51.1±0.5	0.091	<0.001
BMI (kg/m^2)	22.5±3.4	22.3±3.0	24.3±3.4	0.001	<0.001
SBP (mmHg)	112.0±12.0	135.8±17.7	177.7±21.6	<0.001	<0.001
DBP (mmHg)	68.2±9.0	77.9±11.5	105.1±13.5	<0.001	<0.001
Pulse (beats/min)	75.7±12.3	70.9±9.9	77.8±14.0	0.350	<0.001
Creatinine (mg/dL)	0.7±0.2	0.8±0.2	0.7±0.2	0.308	0.003
Total cholesterol (mg/dL)	208.6±45.5	230.7±49.6	220.9±37.5	0.045	0.106
HDL cholesterol (mg/dL)	60.2±18.8		62.6±17.2	0.389	
Uric acid (mg/dL)	5.3±6.8		4.7±1.5	0.440	
Alcohol consumption (%)	21.2	28.9	35.4	0.589	0.090
Smoking (%)	12.1	17.9	30.2	0.065	0.318

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoprotein; NT, normotension; EH, essential hypertension.

The threshold value of the frequencies of the haplotypes included in the analysis was set to $1/2n$ (n: numbers of subjects in each group), as suggested by Excoffier and Slatkin (*25*). All haplotypes below the threshold value were excluded from the analysis. The overall distribution of haplotypes was analyzed using $2 \times m$ contingency tables with a value of $p<0.05$ considered to indicate statistical significance. The p value for each haplotype was determined by the χ^2 analysis and permutation method using the software SNPAlyze version 3.2.3 (*18*, *26*).

Results

Table 1 shows the clinical features for the EH patients and the NT controls. The SBP and DBP were significantly higher in the EH group as compared to those seen in the NT group. Age and serum concentrations of creatinine and uric acid did not differ significantly between the two groups.

We performed an association study using four SNPs. Table 2 shows the genotype and allele distributions of the four SNPs. The overall distributions of the genotypes and alleles did not differ significantly between the EH and NT1 groups. Almost all of the data were duplicated between the groups with the exception of the genotype distribution of rs1047047 between the EH and NT2 group. However, the allelic distribution of rs1047047 was not significantly different between the EH and NT2 group.

LD analysis showed that the four SNPs were located in one haplotype block, because all D' values were beyond 0.5. Because the r^2 between rs883062 and rs885846 was beyond 0.5, the combination of rs883062 and rs885846 was not adequate when using these simultaneously in the one haplotype-based case-control study (Tables 3, 4). These results were completely the same for the NT1 and NT2 groups.

Table 2. Genotype Distribution in Normotensives (NT) and Patients with Essential Hypertension (EH)

	Total subjects					Male subjects					Female subjects				
	NT1	NT2	EH	*p* value *vs*. NT1	*p* value *vs*. NT2	NT1	NT2	EH	*p* value *vs*. NT1	*p* value *vs*. NT2	NT1	NT2	EH	*p* value *vs*. NT1	*p* value *vs*. NT2
Number of participants	279	285	281			180	138	185			99	147	96		
Variants															
rs883062															
Genotype															
C/C	24 (0.086)	24 (0.084)	22 (0.078)			17 (0.094)	13 (0.094)	15 (0.081)			7 (0.070)	11 (0.075)	7 (0.073)		
C/T	111 (0.398)	105 (0.368)	121 (0.431)			73 (0.406)	45 (0.326)	79 (0.427)			38 (0.384)	60 (0.408)	42 (0.438)		
T/T	144 (0.516)	156 (0.547)	138 (0.491)	0.727	0.318	90 (0.500)	80 (0.580)	91 (0.492)	0.861	0.182	54 (0.545)	76 (0.517)	47 (0.490)	0.727	0.901
Allele															
C	159 (0.285)	153 (0.268)	165 (0.294)			107 (0.297)	71 (0.257)	109 (0.295)			52 (0.263)	82 (0.279)	56 (0.292)		
T	399 (0.715)	417 (0.732)	397 (0.706)	0.750	0.346	253 (0.703)	205 (0.743)	261 (0.705)	0.938	0.295	146 (0.737)	212 (0.721)	136 (0.708)	0.522	0.761
rs1047047															
Genotype															
A/A	93 (0.333)	85 (0.298)	90 (0.320)			60 (0.333)	40 (0.290)	61 (0.330)			33 (0.333)	45 (0.306)	29 (0.302)		
A/G	132 (0.473)	146 (0.512)	115 (0.409)			85 (0.472)	68 (0.493)	73 (0.395)			47 (0.475)	78 (0.531)	42 (0.438)		
G/G	54 (0.194)	54 (0.190)	76 (0.270)	0.085	0.023*	35 (0.194)	30 (0.217)	51 (0.276)	0.147	0.200	19 (0.192)	24 (0.163)	25 (0.260)	0.519	0.154
Allele															
A	318 (0.570)	316 (0.554)	295 (0.525)			205 (0.569)	148 (0.536)	195 (0.527)			113 (0.571)	168 (0.571)	100 (0.521)		
G	240 (0.430)	254 (0.446)	267 (0.475)	0.131	0.320	155 (0.431)	128 (0.464)	175 (0.473)	0.250	0.817	85 (0.429)	126 (0.429)	92 (0.479)	0.323	0.273
rs2297566															
Genotype															
A/A	12 (0.043)	13 (0.046)	10 (0.036)			5 (0.028)	4 (0.029)	7 (0.038)			7 (0.070)	9 (0.061)	3 (0.031)		
A/G	94 (0.337)	99 (0.347)	87 (0.310)			63 (0.350)	48 (0.348)	55 (0.297)			31 (0.313)	51 (0.347)	32 (0.333)		
G/G	173 (0.620)	173 (0.607)	184 (0.655)	0.676	0.478	112 (0.622)	86 (0.623)	123 (0.665)	0.516	0.598	61 (0.616)	87 (0.592)	61 (0.635)	0.456	0.530
Allele															
A	118 (0.211)	125 (0.219)	107 (0.190)			73 (0.203)	56 (0.203)	69 (0.186)			45 (0.227)	69 (0.235)	38 (0.198)		
G	440 (0.789)	445 (0.781)	455 (0.810)	0.379	0.228	287 (0.797)	220 (0.797)	301 (0.814)	0.578	0.601	153 (0.773)	225 (0.765)	154 (0.802)	0.479	0.339
rs885846															
Genotype															
G/G	30 (0.108)	26 (0.091)	28 (0.100)			21 (0.117)	17 (0.123)	19 (0.103)			9 (0.091)	9 (0.061)	9 (0.094)		
G/A	125 (0.448)	126 (0.442)	123 (0.438)			82 (0.456)	51 (0.370)	84 (0.454)			43 (0.434)	75 (0.51)	39 (0.406)		
A/A	124 (0.444)	133 (0.467)	130 (0.463)	0.896	0.943	77 (0.428)	70 (0.507)	82 (0.443)	0.899	0.311	47 (0.475)	63 (0.429)	48 (0.500)	0.923	0.245
Allele															
G	185 (0.332)	178 (0.312)	179 (0.319)			124 (0.344)	85 (0.308)	122 (0.330)			61 (0.308)	93 (0.316)	57 (0.297)		
A	373 (0.668)	392 (0.688)	383 (0.681)	0.641	0.822	236 (0.656)	191 (0.692)	248 (0.670)	0.674	0.558	137 (0.692)	201 (0.684)	135 (0.703)	0.810	0.650

*Significant difference in distribution.

Table 3. Pairwise Linkage Disequilibrium in GUCA2B Gene, Evaluated by *D′* and r^2

NT1

		D'			
	SNP	rs883062	rs1047047	rs2297566	rs885846
r^2	rs883062		0.917	0.937	0.950
	rs1047047	0.253		1.000	0.967
	rs2297566	0.094	0.202		1.000
	rs885846	0.725	0.350	0.133	

NT2

		D'			
	SNP	rs883062	rs1047047	rs2297566	rs885846
r^2	rs883062		0.850	1.000	0.909
	rs1047047	0.213		0.973	0.983
	rs2297566	0.103	0.214		1.000
	rs885846	0.668	0.353	0.128	

|D'| >0.5
r^2 >0.5

Table 4. Distribution of Individual Haplotypes

Combination of SNPs	Overall distribution		Distribution of individual haplotypes					
	p value *vs*. NT1	*p* value *vs*. NT2	Haplotype	NT1	NT2	EH	*p* value *vs*. NT1	*p* value *vs*. NT2
rs883062-rs1047047	<0.001*	<0.001*	T-A	0.426	0.439	0.447	0.482	0.780
			C-G	0.279	0.255	0.266	0.603	0.698
			T-G	0.295	0.306	0.259	0.186	0.084
			C-A	0.000	0.000	0.028	0.00006*	0.00007*
rs883062-rs1047047-rs2297566	<0.001*	<0.001*	T-A-G	0.427	0.432	0.438	0.685	0.837
			C-G-G	0.275	0.258	0.260	0.601	0.942
			T-G-A	0.211	0.222	0.185	0.291	0.126
			T-G-G	0.088	0.088	0.082	0.677	0.715
			C-A-G	0.000	0.000	0.035	0.00001*	0.00001*
rs883062-rs2297566	0.629	0.361	T-G	0.507	0.512	0.520	0.677	0.806
			C-G	0.282	0.268	0.293	0.699	0.359
			T-A	0.210	0.219	0.188	0.337	0.184
rs1047047-rs2297566-rs885846	0.656	0.703	A-G-A	0.428	0.442	0.464	0.234	0.462
			G-G-G	0.329	0.313	0.318	0.697	0.843
			G-A-A	0.212	0.219	0.191	0.377	0.244
			G-G-A	0.031	0.027	0.027	0.743	0.934
rs1047047-rs2297566	0.356	0.422	A-G	0.430	0.444	0.471	0.166	0.353
			G-G	0.358	0.338	0.341	0.530	0.929
			G-A	0.212	0.218	0.188	0.331	0.210
rs1047047-rs885846	0.378	0.516	A-A	0.428	0.444	0.468	0.186	0.424
			G-G	0.329	0.312	0.316	0.637	0.877
			G-A	0.243	0.245	0.217	0.300	0.264
rs2297566-rs885846	0.484	0.474	G-A	0.457	0.468	0.491	0.253	0.445
			G-G	0.332	0.312	0.319	0.641	0.822
			A-A	0.212	0.219	0.190	0.379	0.229

*Significant difference in distribution. SNP, single nucleotide polymorphism; NT, normotension; EH, essential hypertension.

The results of the LD analysis provided an estimate of seven combinations of SNPs for the haplotype-based case-control studies (Table 4). Two combinations, rs883062-rs1047047 and rs883062-rs1047047-rs2297566, showed significant differences in overall distributions in the haplotype-based case-control studies. The occurrence of the C-A haplotype at rs883062-rs1047047 was significantly higher in the EH group (2.8%) than in the NT1 group (0.0%) ($p=0.00006$).

The occurrence of the C-A haplotype was not significantly different between EH males (2.9%) and EH females (2.7%) ($p=0.803$). The occurrence of the C-A-G haplotype at rs883062-rs1047047-rs2297566 was significantly higher in the EH group (3.5%) than in the NT group (0.0%) ($p=0.00001$). The occurrence of the C-A-G haplotype was not significantly different between EH males (3.3%) and EH females (4.0%) ($p=0.575$). The results for the second case-control study were the same as those found for the haplotype-based case-control study (Table 4).

As estimated based on the diplotype analysis, there were 10 patients with the C-A haplotype and 16 patients with the C-A-G haplotype in the EH group. The SBP in patients with and without the C-A haplotype were 183.1±34.6 and 170.5±19.7 mmHg ($p=0.053$), respectively. The DBP in patients with the C-A haplotype (114.2±17.1 mmHg) was significantly higher than that in patients without the C-A haplotype (103.3±15.2 mmHg) ($p=0.027$). Other clinical parameters were not significantly different between the patients with and without the C-A haplotype (data not shown). There were no significant differences in any of the clinical parameters between the patients with and without the C-A-G haplotype (data not shown).

Discussion

Initially, it was considered that the physiologic role of uroguanylin was to regulate the secretion of fluid and electrolytes in the intestinal epithelium. While there may be some residual belief that guanylin peptides influence cellular functions *via* the intracellular second messenger, cyclic GMP (cGMP), uroguanylin appears to cause no abnormalities of intestinal fluid secretion in mice lacking GC-C (*1*). However, it should be noted that GC-C, the so-called uroguanylin receptor, is not identical to the natriuretic peptide type C receptor (NPR3). The gene encoding human GC-C (GUCY2C) is located on 12p12, while the human NPR3 gene is located on 5p14-p13. Recently, it was reported that uroguanylin regulates transport in the cortical collecting duct that is independent of GC-C (*27*). These findings suggest that there may be other physiologic roles for uroguanylin in addition to signal transduction *via* GC-C. Although there have been some reports showing that natriuretic peptides and their receptors are related to the pathophysiology of hypertension, there have been no previous association studies concerning the EH and the GUCA2B gene. Our study is the first to attempt to elucidate this relationship. We examined the association between the GUCA2B gene and EH using four SNPs of this gene. The use of SNPs is a valuable tool for association studies examining genomic markers. Since the SNPs consisted of three genotypes and two alleles, we were able to investigate the association between EH and the frequency of the genotype and the allele.

Since the completion in 2001 of the draft sequence of the human genome, the methodology and strategy for doing genetic research have changed dramatically. SNPs are now used for the positional cloning of susceptibility genes by performing whole genome-wide scanning (*28*). However, one of the biggest changes involves haplotype analysis. Recent studies have shown that the human genome has a haplotype block structure that can be divided into discrete blocks of limited haplotype diversity. In each block, a small fraction of SNPs, referred to as "tag SNPs," can be used to distinguish a large fraction of the haplotypes. These tag SNPs have the potential to be extremely useful in association studies, since they make it unnecessary to have to genotype all of the SNPs. The online information of the HapMap projects indicate that the rs883062 and rs885846 in the four SNPs used in our experiment were registered, and the r^2 between rs883062 and rs885846 was 0.631. Therefore, a combination of the two SNPs is not adequate when using these simultaneously in a one haplotype-based case-control study, as was determined in our study.

Haplotype-based analysis is considered to be much more powerful than a marker-by-marker analysis (*29*). In genes with multiple susceptibility alleles, in particular when the LD between the polymorphisms is weak, a haplotype-based association study has advantages over an analysis that is just based on individual polymorphisms (*30*). In the present study, we estimated haplotypes using three polymorphisms (rs883062, rs1047047, rs2297566), which were based on the results of the LD analysis. We used these to conduct a haplotype-based case-control study between the EH and NT groups. Statistically, the two haplotypes were significantly more frequent in the EH group than in the control group. Furthermore, the DBP in patients with the C-A haplotype was significantly higher than that in patients without the haplotype. This is very interesting because a possible functional mutation in the GUCA2B gene linked to the C-A haplotype may be involved in the pathophysiology of EH. Blood pressure may be higher in patients with than in those without the C-A haplotype. Thus, this phenotype could be one of the characteristics of the GUCA2B gene–related hypertension. However, the frequency of the C-A-G haplotype at rs883062-rs1047047-rs2297566 in the EH group was only 3.5%. This is not surprising given the low frequency of the susceptibility haplotype, since EH is thought to be a multifactorial disorder. Uroguanylin belongs to a group of depressor factors that include natriuretic peptides, the nitric oxide system, the adrenomedullin system and prostacyclin. It is thought that each susceptibility gene is related to the development of EH even though the contribution of each gene is small and that these genes may interact in a complex manner. At the present time, it is impossible to predict the contribution of SNPs or haplotypes in the GUCA2B gene in regard to EH. However, the accumulation of additional data on the genotyping of multiple susceptibility genes for EH may ultimately lead to the ability to determine the individual contributions.

In conclusion, the present haplotype-based case-control study showed that the GUCA2B gene could be the susceptibility gene of EH. The present data indicate that the GUCA2B

gene is a promising candidate for use as a genetic marker for EH. Further studies are needed to clarify the causal/susceptibility mutation of the GUCA2B gene and/or neighboring genes in EH.

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References

1. Lorenz JN, Nieman M, Sabo J, *et al*: Uroguanylin knockout mice have increased blood pressure and impaired natriuretic response toenteral NaCl load. *J Clin Invest* 2003; **112**: 1244–1254.
2. Hamra FK, Forte LR, Eber SL, *et al*: Uroguanylin: structure and activity of a second endogenous peptide that stimulates intestinal guanylate cyclase. *Proc Natl Acad Sci U S A* 1993; **90**: 10464–10468.
3. Schulz S, Green CK, Yuen PS, *et al*: Guanylyl cyclase is a heat-stable enterotoxin receptor. *Cell* 1990; **63**: 941–948.
4. Forte LR, Fan X, Hamra FK: Salt and water homeostasis: uroguanylin is a circulating peptide hormone with natriuretic activity. *Am J Kidney Dis* 1996; **28**: 296–304.
5. Forte LR, London RM, Freeman RH, *et al*: Guanylin peptide: renal actions mediated mediated by cyclic GMP. *Am J Physiol Renal Physiol* 2000; **278**: F180–F191.
6. Forte LA, London RM, Krause WJ, *et al*: Mechanisms of guanylin action *via* cyclic GMP in the kidney. *Annu Rev Physiol* 2000; **62**: 673–695.
7. Hess R, Kuhu M, Schulz-Knappe P, *et al*: GCAP-II: isolation and characterization of the circulating form of human uroguanylin. *FEBS Lett* 1995; **374**: 34–38.
8. Martin S, Adermann K, Forssmann WG, *et al*: Regulated, side-directed secretion of proguanylin from isolated rat colonic mucosa. *Endocrinology* 1999; **140**: 5022–5029.
9. Carrithers SL, Jackson BA, Cai WY, *et al*: Site-specific effects of dietary salt intake on guanylin and uroguanylin mRNA expression in rat intestine. *Regul Pept* 2002; **107**: 87–95.
10. Potthast R, Ehler E, Scheving LA, *et al*: High salt intake increases uroguanylin expression in mouse kidney. *Endocrinology* 2001; **142**: 3087–3097.
11. Kinoshita H, Fujimoto S, Nakazato M, *et al*: Urine and plasma levels of uroguanylin and its molecular forms in renal diseases. *Kidney Int* 1997; **52**: 1028–1034.
12. Kinoshita H, Fujimoto S, Fukae H, *et al*: Plasma and urine levels of uroguanylin, a new natriuretic peptide, in nephrotic syndrome. *Nephron* 1999; **81**: 160–164.
13. Carrithers SL, Eber SL, Forte LR, *et al*: Increased urinary excretion of uroguanylin in patients with congestive heart failure. *Am J Physiol Heart Circ Physiol* 2000; **278**: H538–H547.
14. Miyazato M, Nakazato M, Matsukura S, *et al*: Genomic structure and chromosomal localization of human uroguanylin. *Genomics* 1997; **43**: 359–365.
15. Miyazato M, Nakazato M, Yamaguchi H, *et al*: Cloning and characterization of a cDNA encoding a precursor for human uroguanylin. *Biochem Biophys Res Commun* 1996; **219**: 644–648.
16. Nakazato M, Yamaguchi H, Date Y, *et al*: Tissue distribution, cellular source, and structural analysis of rat immunoreactive uroguanylin. *Endocrinology* 1998; **139**: 5247–5254.
17. Dominiczak AF, Negrin DC, Clark JS, *et al*: Genes and hypertension: from gene mapping in experimental models to vascular gene transfer strategies. *Hypertension* 2000; **35**: 164–172.
18. Morita A, Nakayama T, Doba N, Hinohara S, Soma M: Polymorphism of the C reactive protein gene is related to serum CRP level and arterial pulse wave velocity in healthy elderly Japanese. *Hypertens Res* 2006; **29**: 323–331.
19. Nakayama T, Soma M, Mizutani Y, *et al*: A novel missense mutation of exon 3 in the type A human natriuretic peptide receptor gene: possible association with essential hypertension. *Hypertens Res* 2002; **25**: 395–401.
20 Hasimu B, Nakayama T, Mizutani Y, *et al*: A novel variable number of tandem repeat polymorphism of the renin gene and essential hypertension. *Hypertens Res* 2003; **26**: 473–477.
21. Aoi N, Soma M, Nakayama T, *et al*: Variable number of tandem repeat of the 5′-flanking region of type-C human natriuretic peptide receptor gene influences blood pressure levels in obesity-associated hypertension. *Hypertens Res* 2004; **27**: 711–716.
22. Kobayashi Y, Nakayama T, Sato N, Izumi Y, Kokubun S, Soma M: Haplotype-based case-control study of adrenomedullin genes on proteinuria in the subjects with essential hypertension. *Hypertens Res* 2005; **28**: 229–236.
23. Dempster AP, Laird NM, Rubin DB: Maximum likelihood from in complete data *via* the EM algorithm. *J R Stat Soc* 1977; **39**: 1–22.
24. Nakayama T, Soma M, Takahashi Y, *et al*: Association analysis of CA repeat polymorphism of the endothelial nitric oxide synthase gene with essential hypertension in Japanese. *Clin Genet* 1997; **51**: 26–30.
25. Excoffier L, Slatkin M: Maximum-likelihood estimation of molecular haplotype frequencies in a diploid population. *Mol Biol Evol* 1995; **12**: 921–927.
26. Kaneko Y, Nakayama T, Saito K, *et al*: Relationship between the thromboxane A_2 receptor gene and susceptibility to cerebral infarction. *Hypertens Res* 2006; **29**: 665–671.
27. Sindic A, Velic A, Basoglu C, *et al*: Uroguanylin and guanylin regulate transport of mouse cortical collecting duct independent of guanylate cyclase C. *Kidney Int* 2005; **68**: 1008–1017.
28. Ozaki K, Ohnishi Y, Iida A, *et al*: Functional SNPs in the lymphotoxin-alpha gene that are associated with susceptibility to myocardial infarction. *Nat Genet* 2002; **32**: 650–654.
29. Zhang K, Calabrese P, Nordborg M, Sun F: Haplotype block structure and its applications to association studies: power and study designs. *Am J Hum Genet* 2002; **71**: 1386–1394.
30. Morris RW, Kaplan NL: On the advantage of haplotype analysis in the presence of multiple disease susceptibility alleles. *Genet Epidemiol* 2002; **23**: 221–233.