Original Article

Haplotype-Based Case-Control Study Revealing an Association between the Adrenomedullin Gene and Proteinuria in Subjects with Essential Hypertension

Yujin KOBAYASHI^{*1}, Tomohiro NAKAYAMA^{*2}, Naoyuki SATO^{*3}, Yoichi IZUMI^{*4},
Shinichiro KOKUBUN^{*2}, and Masayoshi SOMA^{*4}

Adrenomedullin (AM) has various physiological actions on the cardiovascular system, including vasodilation, diuresis, natriuresis, inhibition of aldosterone secretion, and increases of the cardiac output, all of which cause hypotension. Since AM plays a role in the pathophysiology of vascular diseases, genes controlling AM might be involved in the development and etiology of essential hypertension (EH). However, there have been few studies examining the relationship between the AM gene and hypertension. The aims of this study were to genotype some of the genetic markers for the human AM gene in Japanese subjects, and *via* a haplotype-based case-control study, assess the association between the AM gene and EH or its risk factors, such as hyperlipidemia, renal damage, and proteinuria. We genotyped 205 EH patients and 210 age-matched normotensive (NT) individuals for two single nucleotide polymorphisms of rs4399321, rs7944706 and a microsatellite polymorphism located approximately 5,400 base pairs downstream of the 3' end of the human AM gene. The overall distribution in each variant and haplotype did not significantly differ between the two groups. However, after dividing the groups into those subjects with and without proteinuria, the haplotype analysis revealed a positive association. In conclusion, a possible mutation linked to the haplotype may indicate a genetic predisposition for proteinuria in EH. (*Hypertens Res* 2005; 28: 229–236)

Key Words: haplotypes, adrenomedullin, hypertension, essential, polymorphism

Introduction

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. More than 80% of hypertensive cases are diagnosed as cases of essential hypertension (EH). EH is likely to be a

polygenic disorder that results from the inheritance of a number of susceptibility genes. The causal genes identified probably contribute to between 30% and 50% of the variation in blood pressure seen among individuals (1). These genetic determinants interact with environmental factors such as dietary salt to produce the final disease phenotype. Despite significant recent progress in genomic and statistical tools, the genetic dissection of human EH remains a major chal-

From ^{*1}Nihon University School of Medicine, Tokyo, Japan; and ^{*2}Division of Receptor Biology and ^{*3}Division of Genomic Epidemiology and Clinical Trials, Advanced Medical Research Center and ^{*4}Division of Nephrology and Endocrinology, Department of Medicine, Nihon University School of Medicine, Tokyo, Japan.

^{*1}Sixth grade student.

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Address for Reprints: Tomohiro Nakayama M.D., Ph.D., Division of Receptor Biology, Advanced Medical Research Center, Nihon University School of Medicine, Ooyaguchi-kamimachi, 30–1 Itabashi-ku, Tokyo 173–8610, Japan. E-mail: tnakayam@med.nihon-u.ac.jp

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Fig. 1. Organization of the human AM gene and location of SNPs and the microsatellite based on a haplotype-based case-control study. Boxes indicate exons and lines indicate introns. Closed boxes indicate coding regions.

Table 1. Characteristics of Study Participants

	Total			Men			Women		
	NT	EH	<i>p</i> value	NT	EH	<i>p</i> value	NT	EH	<i>p</i> value
Number of subjects	210	205		147	120		63	85	
Age (years)	52.5±7.2	51.5±4.8	0.104	52.0±6.7	51.2±5.2	0.272	53.6±8.3	51.9±4.0	0.116
BMI (kg/m ²)	23.2±3.1	23.8±2.9	0.168	23.5±2.9	23.9±2.5	0.278	22.6±3.6	23.7±3.3	0.064
SBP (mmHg)	113.3±10.2	171.6±17.7	<0.001	113.4±9.9	169.1±17.3	<0.001	113.2±11.1	175.2±17.6	<0.001
DBP (mmHg)	70.2±8.0	104.8±12.6	<0.001	70.4±7.8	105.7±13.1	<0.001	69.9±8.5	103.5±11.8	<0.001
Pulse (beats/min)	73.6±10.3	76.4±14.0	0.055	70.4±7.8	75.6±13.7	0.331	73.2±9.0	77.4±14.3	0.073
Creatinine (mg/dl)	0.84±0.23	0.83±0.25	0.470	0.92±0.22	0.93±0.24	0.755	0.67±0.15	0.68±0.16	0.723
Proteinuria (%)	9.8	13.8	0.240	12.4	16.5	0.376	3.5	10	0.156
Total cholesterol (mg/dl)	201.6±43.5	208.7±38.1	0.089	196.7±41.8	201.1±38.7	0.393	213.0±45.7	218.7±35.0	0.397
HDL cholesterol (mg/dl)	56.0±16.6	59.1±17.1	0.086	53.9±15.6	55.7±17.0	0.432	61.0±17.9	63.6±16.3	0.401
Uric acid (mg/dl)	5.9±4.8	5.4±1.6	0.086	6.0±1.4	5.9±1.4	0.737	5.7±8.6	4.7±1.6	0.310
Alcohol consumption (%)	61.4	66.1	0.379	76.1	85.6	0.084	33.3	38.5	0.562
Smoking (%)	39.9	54.2	0.010	50	67.5	0.011	20.8	34.6	0.094

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

lenge (2).

Adrenomedullin (AM) is a 52-amino acid peptide that was originally isolated from a human pheochromocytoma in 1993 (3). Plasma AM levels are increased in patients with cardiovascular diseases such as myocardial infarction, pulmonary hypertension, heart failure, chronic renal failure and EH (4). Because the AM concentration and gene expression levels are quite high in endothelial cells, AM might be secreted mainly by vascular endothelial and smooth muscle cells (5). AM has various physiological effects on the cardiovascular system, including vasodilatation (6), diuresis, natriuresis, inhibition of aldosterone secretion and increases of the cardiac output (7), all of which cause hypotension. Furthermore, AM protects against cardiac hypertrophy, fibrosis, and renal damage, which are known to be complications of hypertension (8). Thus AM plays a role in the pathophysiology of vascular dis-

eases, and the genes that control this system may be involved in the development of hypertension (9). The human AM gene is located on 11p, spans approximately 2,400 base-pairs (bp) with 4 exons and 3 introns and has TATA, CAAT and GC boxes in the 5'-flanking region (10). Although many research groups have investigated the gene expression and regulation of AM and its receptors (11), there are few studies that have examined the relationship between the AM gene and EH (12). To the best of our knowledge, there have been no reports that have determined the haplotypes of the AM gene or a haplotype-based case-control study for EH.

The aims of this study were to genotype some of the genetic markers of the human AM gene in Japanese subjects, and *via* a haplotype-based case-control study, assess the association between the AM gene and EH or its risk factors, such as hyperlipidemia, renal damage, and proteinuria.

Table 2. Genotype Distribution in NT Subjects and Patients with EH

Variants		NT (210)*	EH (205)*	<i>p</i> value
rs4399321	Genotype			
	AA	113 (0.538)	110 (0.53.7)	
	AG	79 (0.376)	78 (0.380)	
	GG	18 (0.086)	17 (0.083)	0.993
	Allele			
	A	305 (0.726)	298 (0.727)	
rs7944706	Genotype			
	GG	85 (0.405)	78 (0.381)	
	AG	88 (0.419)	98 (0.478)	
	AA	37 (0.176)	29 (0.141)	0.417
	Allele			
	G	258 (0.614)	254 (0.620)	
Microsatellite	Genotype			
	11, 11	18	16	
	11, 13	32	34	
	11, 14	39	42	
	11, 15	1	1	
	11, 19	4	3	
	13, 13	19	13	
	13, 14	44	44	
	13, 15	1	2	
	13, 19	3	6	
	14, 14	42	35	
	14, 19	7	9	
	Allele			
	11	112	112	
	13	118	112	
	14	174	165	
	15	2	3	
	19	14	18	0.914

NT, normotension; EH, essential hypertension. *Number of participants.

Methods

Subjects

A group of 205 patients were diagnosed with EH according to the following criteria: a seated systolic blood pressure (SBP) above 160 mmHg and/or diastolic blood pressure (DBP) above 100 mmHg that occurred on 3 occasions within 2 months after the first medical examination. None of the patients were using any anti-hypertensive medication. Subjects diagnosed with secondary hypertension were excluded. We also included 210 normotensive (NT) healthy individuals as controls. None of the NT participants had any family history of hypertension, and they all had SBP and DBP below

130 and 85 mmHg, respectively. A family history of hypertension was defined as 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 according to a protocol approved by the Human Studies Committee of Nihon University (13).

Biochemical Analysis

Plasma concentrations of total cholesterol and high-density lipoprotein (HDL)-cholesterol and serum concentrations of creatinine and uric acid were measured using the methods of the Clinical Laboratory Department of Nihon University Hospital (14). Proteinuria (PU) was diagnosed by dipstick analy-

Table 3. Haplotype Distribution in NT Controls and EH Patients

Haplotype	NT (420)	EH (410)
A-A-14	0.267	0.249
A-G-13	0.190	0.185
A-G-11	0.181	0.188
G-G-11	0.081	0.078
G-A-14	0.081	0.098
G-G-13	0.076	0.083
A-G-14	0.055	0.051
A-A-13	0.014	0.005
G-G-19	0.014	0.007
A-A-19	0.012	0.015
G-G-14	0.012	0.005
A-G-19	0.003	0.022
A-A-11	0.002	0.007
A-A-15	0.002	0.005
G-A-11	0.002	0.000
G-A-15	0.002	0.002
G-A-19	0.002	0.000

$\chi^2=13.3$, $p=0.501$. Haplotypes are shown as combined alleles of SNPs and repeat number of the microsatellite. NT, normotension; EH, essential hypertension; SNP, single nucleotide polymorphism.

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Genotyping

Using information about allelic frequencies of single nucleotide polymorphisms (SNPs) registered on the website of the National Center for Biotechnology Information (NCBI) and Celera Discovery System (Applied Biosystems, Branchburg, USA), two SNPs around the human AM gene with minor allele frequencies greater than 20% were selected. SNPs with relatively high minor allele frequencies have been shown to be very useful as genetic markers for genetic association studies. These two SNPs were confirmed using the NCBI website, and had the accession numbers rs4399321 and rs7944706 (Fig. 1). The rs4399321 SNP was located at -3053 nucleotides (nt) upstream of the transcription initiation site and is responsible for the changing of adenine to guanine. The rs7944706 was located at +2367 nt downstream of the guanine of the terminal codon and is responsible for the changing of guanine to adenine. Genotypes were determined using Assays-on-Demand kits (Applied Biosystems) together with TaqMan® PCR. When allele-specific fluorogenic probes hybridize to the template during the polymerase chain reaction (PCR), the 5' nuclease activity of *Taq* polymerase can discriminate 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 is labeled with a reporter dye (VIC and FAM) at the 5' end and TAMRA at the 3' end. Amplification by PCR was performed using TaqMan® Universal Master Mix (Applied Biosystems) with a 25 µl reaction volume that contained a final concentration of 50 ng DNA, 700 nmol/l primer, and 100 nmol/l probe. Thermal cycling conditions consisted of 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 62°C for 1 min and were performed on a GeneAmp 9700® system.

All 96-well plates contained 80 samples of unknown genotype, six known allele 1 homozygotes, six known allele 2 homozygotes, and four reactions with reagents but no DNA. The homozygote and control samples without DNA were required for the SDS 7700® signal processing outlined in the TaqMan® Allelic Discrimination Guide (Applied Biosystems). Direct sequencing, single-strand conformation polymorphism (SSCP), or denaturing high pressure liquid chromatography was used to confirm control sample genotypes. PCR plates were read on the SDS 7700® instrument in the endpoint analysis mode of the SDS version 1.6.3 software package (Applied Biosystems). Genotypes were visually determined by comparison with the dye-component fluorescent emission data shown in the XY scatterplot of the SDS software. Genotypes were also automatically determined by the signal processing algorithms in the software. Results of both scoring methods were saved to two output files for later comparison.

The microsatellite polymorphism was located approximately 5,400 bp downstream of the 3' end of the human AM gene. This microsatellite is a GT repeat that was found in the database of the human genome, although it had not yet been registered in the NCBI and Celera Discovery System. Ishimitsu *et al.* previously reported that this microsatellite is associated with EH (12). The nucleotide sequences of the PCR primers were designed to be the same as those of the primers set by Ishimitsu *et al.* (12). The 5' terminal of the forward primer was dye-conjugated with FAM (Applied Biosystems). The reaction mix for PCR was prepared as previously described (13). Thermal cycling conditions were 96°C for 3 min, and then 35 cycles of 98°C for 25 s, 60°C for 30 s, and 72°C for 45 s. Thermal cycling was performed on a GeneAmp 9700® system. The PCR products were loaded on an ABI 3700® DNA analyzer (Applied Biosystems) for the genotyping as described previously (15). The fluorescent signal was recorded and analyzed by the GeneScan version 2.1 software. Fragments from reactions using each of the different fluorescent dyes were plotted separately, and the sizes of fluorescent peaks were estimated for the base pairs by referencing the in-lane size standard. Marker alleles were classified according to their size using the Genotyper version 2 software. In addition to the automated allele calling, we performed manual surveillance of all genotypes (15).

Table 4. Characteristics of Study Participants in Each Genotype

Microsatellite	Subjects without 19-repeat allele	Subjects with 19-repeat allele	<i>p</i> value
Number of subjects	383	32	
Age (years)	52.0±6.2	52.4±6.2	0.702
BMI (kg/m ²)	23.5±3.0	23.6±2.6	0.813
SBP (mmHg)	141.9±32.9	144.7±27.9	0.642
DBP (mmHg)	87.0±20.4	90.8±17.5	0.310
Pulse (beats/min)	75.3±12.6	74.2±12.0	0.663
Creatinine (mg/dl)	0.83±0.23	0.89±0.30	0.185
Proteinuria (%)	25.9	10.5	0.016*
Total cholesterol (mg/dl)	205.0±41.5	206.2±36.2	0.876
HDL cholesterol (mg/dl)	57.3±16.6	60.4±20.5	0.352
Uric acid (mg/dl)	5.5±1.5	5.6±1.5	0.686
Alcohol consumption (%)	63.8	68.2	0.682
Smoking (%)	48.2	47.8	0.972

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

Haplotype Analysis

Based on the genotype data of the three genetic variations, the frequency of the haplotype was estimated using the expectation/maximization (EM) algorithm estimate (16). For determining the haplotype, SNPalyze ver. 4.0 was used (DYNACOM Co., Ltd., Yokohama, Japan), and is available from the website <http://www.dynacom.co.jp/products/package/snpalyze/index.html> (17).

Statistical Analysis

Data are shown as the mean±SD. Differences in clinical data 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. The overall distribution of SNP alleles was analyzed by 2×2 contingency tables, and the distribution of the SNP genotypes between EH patients and NT controls was tested using a 2-sided Fisher exact test and multiple logistic regression analysis. Statistical significance was established at $p<0.05$.

The overall distribution of microsatellite alleles between EH patients and NT subjects was analyzed by 2×*n* contingency tables (18), and *p* values less than 0.05 were considered significant. Individual differences in allele frequencies were tested using 2×2 contingency tables for each allele and the combined remaining alleles, and a *p* value of less than 0.05/*n* was considered significant to correct for the number of comparisons made (Bonferroni correction) (18). The threshold value of frequencies of haplotypes included in the analysis was set to 1/2*n* (where *n* is the number of subjects in each group), as suggested by Excoffier and Slatkin (19). All haplotypes below the threshold value were excluded from the analysis. The overall distribution of haplotypes was analyzed

using 2×*m* contingency tables with a value of $p<0.05$ considered to indicate statistical significance. The level of *p* used for significance was determined by the 0.05/*m* number (20). For the permutation method, values of $p<0.05$ were considered to indicate statistical significance (21).

Results

Table 1 shows the clinical features of the EH patients and NT controls. The SBP and DBP were significantly higher in the EH than in the NT group. Age, body mass index (BMI), pulse rate, serum concentrations of creatinine, and plasma concentrations of total cholesterol and uric acid did not significantly differ between the two groups.

We performed an association study using three genetic variants. Table 2 shows the distribution of the genotypes and alleles. The overall distribution of genotypes and alleles of rs4399321 and rs7944706 did not significantly differ between the EH and NT groups. The overall distribution of alleles of the microsatellite also did not significantly differ between the EH and NT groups. Table 3 shows the frequencies of 17 observed haplotypes (H1 to H17). The overall distribution of haplotypes did not significantly differ between the EH and NT groups ($\chi^2=13.3$; $p=0.501$).

Ishimitsu *et al.* reported that the frequency of the 19-repeat allele of the microsatellite was significantly higher in EH than NT (12). Therefore, we compared the clinical characteristics in subjects with or without the 19-repeat allele (Table 4). Although there were no significant differences in the SBP and DBP between the two groups, surprisingly, the levels of PU were significantly different between the two groups. Therefore, we performed a haplotype analysis on two subgroups, one in which PU was present and one in which it was absent. As seen in Table 5, the overall distribution of haplotypes showed a significant difference between the EH and NT

Table 5. Haplotype Distribution after Division of the Subjects into Groups with or without PU

Haplotype	Non-PU (638)	PU (84)	χ^2 <i>p</i> value	Permutation <i>p</i> value
A-A-14	0.361	0.321	0.481	0.503
A-G-13	0.269	0.250	0.707	0.730
G-G-11	0.260	0.262	0.972	0.907
A-G-14	0.040	0.071	0.182	0.285
A-G-19	0.027	0.083	0.007*	0.009 [#]
A-A-11	0.010	0.000		0.598
A-A-13	0.010	0.000		0.600
G-G-13	0.006	0.012	0.548	0.756
G-G-19	0.004	0.000		0.390
A-G-11	0.003	0.000		0.545
G-A-14	0.002	0.000		0.449
G-G-14	0.002	0.000		0.770
A-A-15	0.000	0.000		0.389

PU, subjects with proteinuria; Non-PU, subjects without proteinuria. Haplotypes are shown as combined alleles of rs4399321, rs7944706 and repeat number of the microsatellite. *Indicates significant difference after a Bonferroni correction. [#]Indicates significant difference.

groups ($\chi^2=11.8$; $p=0.019$). Furthermore, the A-G-19 haplotype was significantly higher in the PU group by χ^2 test after the Bonferroni correction ($p=0.007$) and by the permutation method ($p=0.009$).

Discussion

Animal model studies have demonstrated that variations in the AM gene affect blood pressure and that the AM gene is directly involved in the development of hypertension. Homozygous AM knockout mice (AM^{-/-}) die with hydrops fetalis and cardiovascular abnormalities (9). Blood pressure is lower and plasma cGMP is higher in transgenic mice overexpressing AM than in wild-type mice (22). Renal perfusion pressure is higher in mice that are heterozygotic for AM (AM^{+/-}) than in wild-type mice and lowest in mice overexpressing AM (23). Loading with angiotensin and salt causes more coronary vascular fibrosis and hyperplasia, left ventricular hypertrophy, increased urinary secretion of markers for oxidative stress and intimal thickening caused by cuff placement in heterozygotic (AM^{+/-}) than in wild-type mice (24, 25). Shindo *et al.* found that arterial blood pressure in unrestrained heterozygous AM knockout mice is significantly higher than that in their wild-type littermates (26).

Ishimitsu *et al.* examined the microsatellite DNA polymorphism lying 3' downstream of the AM gene in normotensives and in patients with EH. They found that among the 4 types of alleles with CA-repeat numbers of 11, 13, 14, and 19, the frequency of the 19-repeat allele was higher in EH than in NT. They hypothesized that the 19-repeat allele is associated with the risk of developing hypertension (12). This particular study implies that the AM gene has a close relationship with the development of hypertension. However, no reports have

described a relationship between the AM gene and EH when using the haplotype of this gene. Therefore, we tried to establish a haplotype using SNPs and a microsatellite in the gene. In our experiment a 15-repeat allele of the microsatellite was discovered to be a novel allele. However, it was of a low frequency and its discovery was not of significance by itself. Our EH case-control study showed a negative association. The discrepancies in the results of the genetic association studies are not problematic because the results sometimes depend on the sample collection conditions and racial differences. Our criteria for EH and NT were more strict than those in the previous report, which included borderline hypertensives in its NT groups (12).

Although the haplotype-based case-control study did not reveal an association with EH, one haplotype was identified as being associated with PU. Ishimitsu *et al.* have also reported that the frequency of the 19-repeat allele of the microsatellite was higher in diabetic patients on hemodialysis than in diabetic patients without nephropathy (27). These results are very interesting because they suggest that the 19-repeat alleles may be related to the factor responsible for the development of diabetic nephropathy. Unfortunately, Ishimitsu *et al.*'s study did not resolve the mechanisms involved in the development of diabetic nephropathy or any further information on PU. The investigators themselves pointed out that the frequency of the 19-repeat allele of the microsatellite was not increased in hypertensive diabetic patients without nephropathy, which decreases the likelihood that the relationship between the 19-repeat allele and diabetic nephropathy is mediated by a predisposition to hypertension. AM has been reported to play a renoprotective role in chronic hypertensive renal failure (28), and the plasma and urinary AM levels in patients with chronic glomerulonephritis have been shown to

reflect the disease activity or glomerular damage represented by the degree of PU (29). Overall these results suggest that AM is involved in the pathophysiology of renal function or PU. Our results in this case-control study for PU are consistent with this hypothesis, and this is the first report showing an association between the AM gene and PU. The haplotype showing positive association may be a genetic marker of PU with EH in Japanese subjects, and may be in strong linkage disequilibrium with another functional DNA variant(s) of the AM gene or neighboring genes.

In conclusion, this is the first study to examine the correlation between the human AM gene and EH by using haplotypes. Although this study did not find a relationship between the AM gene and EH, it did reveal a significant association between the AM gene and PU. The data that can be obtained by examining the genetic information of an individual's AM gene may provide a genetic marker for the PU that is found in EH.

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