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Title page

Alpha-adducin G460W polymorphism, urinary sodium excretion, and blood pressure in community-based samples

running title: ADD1 polymorphism, sodium intake and blood pressure (45 characters)

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Background. There is limited evidence on the gene-environmental interaction among α-adducin G460W gene polymorphism, sodium intake and blood pressure levels among a general population. *A priori* hypothesis is that the association between G460W polymorphism and blood pressure is more evident among persons with higher sodium intake than those with lower sodium intake.

Methods. We conducted a population-based cross-sectional study of 2,823 men and women aged 30-74 years in a Japanese rural community to examined the association of the α-adducin G460W polymorphism with blood pressure levels stratified by salt intake, as estimated by 24-hour urine collection and dietary questionnaire.

Results. There was no difference in systolic or diastolic blood pressure levels among the GG, GW and WW groups for women, but for men, mean systolic blood pressure tended to be higher in WW group than in the GG group. When we stratified men according to sodium excretion/intake, mean systolic blood pressure was significantly higher in the WW group than the GG group among men with higher urinary sodium excretion (138.8 *vs.* 133.6 mmHg, p=0.02) and tend to be higher among men with higher past sodium intake. No genetic association was found among women or among men with lower urinary sodium excretion or lower sodium intake.

Conclusion. The α-adducin WW genotype was associated with higher systolic blood pressure among men with a higher sodium intake. (223 words)

Key words: epidemiology, hypertension, sodium, gene-environment interaction, salt-sensitivity

INTRODUCTION

α-adducin is a cytoskeletal protein that regulates transmembrane ion transport.[1] The genetic association of α-adducin polymorphism with salt sensitive hypertension was first reported in the Milan Hypertensive Strain rats (MHS)[2], and later in Caucasians in Italy and France.[3] That human study showed not only linkage and association between the α-adducin G460W polymorphism (glycine-to-tryptophan substitution) and hypertension, but also a greater decrease in mean blood pressure after Weinberger's salt-sensitivity test or treatment with hydrochlorothiazide in GW hypertension patients than in GG patients. Afterward, several clinical studies showed a significant relationship between α-adducin polymorphism and salt-sensitivity among hypertensive patients.[4,5] However, no population-based study was performed for gene-environment interaction among α-adducin polymorphism, salt intake, and blood pressure.

Therefore, we conducted a large community-based observational study of 2,823 Japanese men and women to carefully examine associations between the α-adducin gene polymorphism and blood pressure levels, stratified by sodium intake. Our *a priori* hypothesis is that persons with the 460W allele of α-adducin gene have elevated blood pressure levels in response to a higher sodium intake, and thus the association between α-adducin polymorphism and blood pressure is more evident among persons with a higher sodium intake than those with a lower sodium intake.

METHODS

Study population

The subjects were residents of the rural farming community of Kyowa, central Japan (the 2000 census population of ages 30-74 years = 9,722). In this community, a general stroke prevention program and annual cardiovascular risk survey have been conducted since 1981.[6] We included in the present study individuals who were aged 30-74 years and participated in the 2001 survey (n=2,972). At the time of the survey, physician epidemiologists explained the protocol to all participants, and obtained written informed consent from 95% (n=2,823) of them. The data of these 2,823 persons were used in the analysis. The study protocol was approved by the Medical Ethics Committee of the University of Tsukuba.

Population surveys

Well-trained blood pressure observers measured arterial systolic blood pressure and fifth-phase diastolic blood pressure using standard mercury sphygmomanometers on the right arm of quietly seated participants after at least a 5-minute rest. When the first systolic blood pressure reading was ≥140 mmHg and/or diastolic blood pressure was ≥90 mmHg, the observers repeated the measurement; the average of first and second readings was used in the analyses, and otherwise the first reading was used. We used body mass index (BMI) and alcohol intake as confounding variables, and urinary sodium excretion estimated by 24-hour urine collection, and past and present sodium intake scores estimated by self-administered questionnaire as effect modifiers. Height without shoes and weight in light clothing were measured and BMI was calculated as weight in kg divided by the square of height in m^2. Interviews were conducted to determine usual weekly ethanol intake in the go unit, a Japanese traditional unit of volume corresponding to 23g ethanol, which was converted to grams of ethanol per day.

All participants in the survey were asked to complete a self-administered questionnaire to estimate both present and past habitual sodium intake. Past sodium intake was defined as the intake before the recognition of hypertension for hypertensives, and for approximately 10 years before the survey for normotensives. A sodium intake score was calculated by adding one point for each of 10 types of sodium intake: *1)* prefer salty-food, *2)* use salty seasoning, *3)* eat two or more miso soup servings per day, *4)* eat pickles two or more times per day, *5)* eat salty pickles, *6)* put soy sauce on pickles, *7)* put soy sauce on meals, *8)* eat salt-preserved food one or more times per week, *9)* eat salty noodle soup, and *10)* do not try to reduce salt intake. This scoring system was previously validated[7,8], and was tested again in the present study. Sex and age-adjusted mean 24-hour sodium excretion values across quintiles of the present sodium intake score (n = 1,674) were 168, 180, 183, 195 and 203 mmol/L (p for trend < 0.0001). Repeatability of the present and past sodium intake scores was also tested previously[7], and was tested again by repeating the questionnaire one to two years apart for a sub-sample (n = 287); the Spearman correlation coefficient was 0.73 for the present sodium intake score ($p<0.0001$), and 0.62 for the past sodium intake score ($p<0.0001$). In the present study, 99% of the subjects completed the sodium intake questionnaire.

To estimate salt intake more accurately, some participants collected one 24-hour urine sample. The participants were asked to collect all urine over 24 hours using a 3- or 4-litre plastic bottle. They were also asked to record the time of each urination, whether they used inappropriate urine collection methods and the number of missed urine collections. For each sample, the total urine volume was measured and transferred into 2-ml plastic vials and stored at –80°C for a month until analysis. Sodium and potassium concentrations were analyzed using an electrolyte analyzer (Hitachi Inflameter 775, Hitachi Ltd., Tokyo, Japan). Urine samples of less than 500 ml or those with incomplete collections based on records were excluded from the analyses. Urine sodium excretion was calculated by the concentration of each electrolyte

(mmol/L) multiplied by the total urine volume (L). We included in the analyses a total of 1,681 subjects who completed the 24-hour urine collection between 1982 and 2002.

DNA genotyping

α-Adducin G460W genotypes were determined by allele-specific PCR method using KOD DNA polymerase (KODplus; Toyobo, Osaka, Japan), as described elsewhere.[9] The designed allele-specific sense primers were ACGAAGCTTCCGAGGAXGG for the 460G allele and GACGAAGCTTCCGAGGAXTG for the 460W allele, and a 5' biotin-end-labeled antisense primer (biotin-CACACCTTAGTCTTCGACTTGG).

Statistical analysis

The analysis of covariance was used to compare sex-specific age-adjusted mean values of risk characteristics according to the genotypes. The chi-square test was used to examine whether the genotype distributions differed from that expected from Hardy-Weinberg equilibrium. The relationship between genotype and blood pressure levels was examined by the Dunnett's t test stratified by the medians of urinary excretion of sodium, and present and past sodium intake scores. We regarded GG genotype as reference for Dunnett's test. The interactions of genotype with age and urinary sodium excretion/intake scores in relation to blood pressure levels were examined using cross-product terms. All statistical analyses were performed using SAS version 8.02 software (SAS Institute Inc., Cary, NC). All p values for statistical tests were two-tailed, and $p<0.05$ was regarded as statistical significance.

RESULTS

The frequencies of α-adducin genotypes were 21.2% (GG), 48.4% (GW), and 30.4% (WW). The genotype distribution was in concordance with Hardy-Weinberg equilibrium for men, women and total subjects. Table 1 shows sex-specific age-adjusted characteristics of each genotype group. None of these characteristics, including antihypertensive medication use, varied among the three genotypes.

The mean values of blood pressure adjusted for sex, age, antihypertensive medication use, BMI, and alcohol consumption among α-adducin genotypes are shown in Table 2. There was no difference in blood pressure levels among the GG, GW and WW groups for women, but for men, mean systolic blood pressure tended to be higher in WW group than in the GG group. This positive association reached statistical significance among older men (≥55 years), and the age interaction was also significant (p=0.006).

When we stratified men by the medians of sodium excretion/intake (Table 3), mean systolic blood pressure was 5.2 mmHg higher in the WW group than the GG group in men with higher urinary sodium excretion (≥189 mmol/day, mean excretion=257 mmol/day) (p=0.02), but not in those with lower excretion (<189 mmol/day, mean excretion=141 mmol/day); the interaction was statistically significant (p=0.03). Mean systolic blood pressure was 4.1 mmHg higher in the WW group than in the GG group among men with higher past sodium intake scores (p=0.08), but not among those with lower scores. However, the interaction did not reach statistical significance. The systolic blood pressure did not differ between GG and WW groups when stratified by present sodium intake score. Instead, mean systolic blood pressure was 3.1 mmHg higher in the GW group than in the GG group in subjects with higher present sodium intake scores (p=0.08); the interaction did not reach statistical significance. These associations became stronger when excluding persons on antihypertensive medication (not shown in the

tables). Mean systolic blood pressure in WW groups *vs.* GG groups were 133.3 *vs.* 130.3 mmHg (p=0.09) for men, 137.4 *vs.* 130.8 mmHg (p=0.01) for men with higher sodium excretion, 130.5 *vs.* 128.1 mmHg (p=0.34) for men with higher present sodium intake score, and 134.0 *vs.* 129.1 mmHg (p=0.06) for men with higher past sodium intake score.

DISCUSSION

Among total samples, we found no significant association between the α-adducin G460W polymorphism and blood pressure levels in either sex. The lack of a significant association between the 460W allele and systolic blood pressure levels overall was not surprising, as this finding was consistent with the results of a large group of previous studies.[10-23] However, among men aged ≥55 years, we found a significant association between the α-adducin G460W genotype and systolic blood pressure levels, which was consistent with a recent study of Italians.[24]

As we hypothesized *a priori*, we found a positive association between the α-adducin G460W genotype and systolic blood pressure levels among men with higher urinary sodium excretion. This finding suggests that the α-adducin genotype may be a genetic risk factor for salt-sensitive hypertension. Cusi *et al.* first showed a positive linkage and association of the α-adducin G460W polymorphism with hypertension[3]: there was also a greater decrease in mean arterial pressure after the salt-sensitivity test and chronic diuretic treatment among hypertensive patients with the GW genotype compared to those with the GG genotype. However, the results of subsequent association studies were inconsistent. Studies in African-Americans, who are believed to have a higher prevalence of salt-sensitive hypertension, did not show any association between α-adducin G460W polymorphism and hypertension.[15,16] On the contrary, among people of South African ancestry, the 460W allele was more frequent among hypertensives than normotensives.[25] Several clinical studies in Japan[26,27], in Italy[4,28,29] and in the United States and France[5], but not in Scotland[10] or Poland[21], found relationships of the G460W polymorphism with lower plasma renin activity and/or salt-sensitivity. Another recent Japanese community-based study did not show any significant association of the α-adducin G460W polymorphism with hypertension or lower plasma renin

activity, but they showed that G460W polymorphism was associated with hypertension only among younger persons (<60 years) with low plasma renin activity (<1.0 ng/ml per h).[22]

Mean urinary sodium excretion in the present study was 186 mmol/day, which was higher than in other studies except one[23]; 140-164 mmol/day in French and Italian hypertensives[3], 151 mmol/day in Scottish subjects[10], 156 mmol/day in American-Caucasians[15], 168 mmol/day in American-Africans[15], 153-172 mmol/day in Italian hypertensives[29] and 165-202 mmol/day in Belgian subjects.[23] Moreover, we observed a relationship between the 460W polymorphism and blood pressure only in the high urinary sodium excretion group (mean = 257 mmol/day). This finding was compatible with results from two prior studies[4,5]. In one study of 108 hypertensives[4], the increase in mean arterial pressure levels after an acute intravenous sodium load (310 mmol over 2 hours) was greater in the GW+WW group than in the GG group (+8.1 *vs.* +2.2 mmHg; p=0.0001) although the baseline mean arterial pressure was not different between the two groups. Correspondingly, the urinary sodium excretion after the sodium load was lower in the GW+WW group than the GG group (296 *vs.* 393 μmol/min; p<0.03). The other study with 279 hypertensives[5] showed that there was a greater systolic blood pressure reduction in response to a reduction in dietary sodium intake from high sodium balance (24-hour urinary sodium excretion of ≥160 mmol/day) to low sodium balance (≤32 mmol/day) in WW (-25 mmHg) than GG (-14 mmHg) or GW (-12 mmHg) hypertensive patients. Our observations and these experimental findings suggest that the G460W polymorphism may be associated with high blood pressure in subjects with a very high sodium intake. Inconsistent results from previous observational studies may be partly due to lower sodium intakes in their study populations. The present large observational study confirmed the genetic association in a general population with a high sodium intake.

The strength of the present study was the use of the largest study population of all reported studies on α-adducin polymorphisms and the availability of 24-hour urine collections

for 60% of the participants and sodium intake scores for all participants. These allowed us to test gene-environmental interactions of α-adducin G460W genotypes with blood pressure levels in a general population. The present study extended the evidence on the gene-environmental interaction, which was suggested by previous clinical studies of hypertensives.[4,5]

There were several limitations in the present study. Firstly, if the G460W polymorphism largely affects the sodium excretion due to a potential effect on enhanced renal sodium reabsorption, the stratification by the sodium excretion may not be adequate because it might be influenced by both sodium intake and the genotype itself. However, the 460W allele reduces approximately 30% of the sodium excretion only after saline loading[4] or high sodium diet[5], but mean sodium excretion did not vary among the genotypes in free-living conditions[4,10,15,29,30] including the present study. Thus, the magnitude of effect on urinary sodium excretion by the genotype itself may be small in the present study. Secondly, the gene-blood pressure associations were not statistically significant when stratified by sodium intake scores, although that association among men with higher past sodium intake score showed a trend towards significance ($p=0.08$). The lack of gene-blood pressure association among men with higher present sodium intake score was in part due to the under-reporting of sodium intake among hypertensives. In this study, the mean present sodium intake score was lower among hypertensives than among normotensives (5.4 *vs.* 5.9, $p<0.0001$), whereas urinary sodium excretion (200.8 *vs.* 197.3 mmol/day, $p=0.56$) and past sodium intake score (6.8 *vs.* 6.9, $p=0.37$) did not differ between the two groups. Thirdly, approximately 20% of the subjects used antihypertensive medications, which may have obscured the genetic effect of blood pressure levels. When we excluded persons on antihypertensive medication from the analyses, the association became somewhat stronger for men with higher sodium excretion and with higher past sodium intake score. Fourthly, we asked systematically a sample of the

participants (about 200 subjects on average) for the urine collection in each annual survey between 1982-2002; of these approximately 80% of the subjects agreed to provide urine collection samples. When we analyzed the gene-blood pressure association according to years between blood pressure measurement and urine collection for men, i.e. <3 years (in 2000-2002: 29%) and ≥3 years (in 1999 or before: 71%), the results did not differ materially; multivariate adjusted mean systolic blood pressure were 133.2mmHg in GG *vs.* 140.1mmHg in WW for men with higher sodium excretion when we used the urine data collected in 2000-2002, multivariate adjusted mean systolic blood pressure were 133.7mmHg in GG *vs.* 137.9mmHg in WW for men with higher sodium excretion when we used the urine data collected in 1999 or before. There was no interaction between these years and genotype in relation to systolic blood pressure levels ($p=0.78$). Lastly, we did not observe any association between the genotype and blood pressure levels among women. A possible reason for the lack of association among women is that a lower mean sodium excretion among women than among men (177.0 mmol/d *vs.* 199.0 mmol/d, $p<0.0001$) may weaken the real association.

In conclusion, the α-adducin WW genotype was associated with higher systolic blood pressure among men with a higher sodium intake estimated by a 24-hour urine collection and dietary questionnaire. Our results support the hypothesis that the α-adducin WW genotype is a genetic risk factor for salt-sensitive hypertension.

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Table 1. Age-adjusted characteristics according to alpha-adducin G460W genotype, men and women aged 30-74 years.

	Women			Men			Total		
	GG	**GW**	**WW**	**GG**	**GW**	**WW**	**GG**	**GW**	**WW**
Number	362	860	553	237	505	306	599	1,365	859
Age, year	57.0	56.5	56.8	59.2	58.5	59.6	57.9	57.3	57.8
Systolic blood pressure, mmHg	131.7	131.3	129.7	133.3	134.9	135.1	132.3	132.7	131.6
Diastolic blood pressure, mmHg	77.3	77.1	76.5	80.9	80.7	81.0	78.7	78.5	78.1
Use of antihypertensive medication, %	21.9	20.4	23.0	25.0	23.7	21.6	23.1	21.6	22.5
Hypertension, %†	40.6	38.1	36.8	47.8	47.4	47.5	43.3	41.6	40.6
Body mass index, kg/m^2	23.7	23.5	23.4	24.0	23.9	23.8	23.8	23.6	23.5
Alcohol intake, g/day	2.0	1.6	1.3	20.8	21.4	20.7	9.4	9.0	8.2
24h urine collection (n)	193	494	318	165	320	191	358	814	509
Urine sodium excretion (mmol)	173.1	177.4	178.8	199.3	196.3	203.1	185.3	185.0	187.7
Present sodium questionnaire completed (n)	361	854	549	235	500	303	596	1,354	852
Present sodium intake score	4.6	4.6	4.7	5.9	5.7	5.5	5.1	5.0	5.0
Past sodium questionnaire completed (n)	359	854	548	233	496	302	592	1,350	850
Past sodium intake score	5.8	5.9	6.0	7.0	6.8	6.7	6.2	6.3	6.2

†Hypertension was defined as systolic blood pressure of ≥ 140 mmHg and/or diastolic blood pressure, of ≥ 90 mmHg and/or use of antihypertensive medication.

Table 2. Blood pressure levels according to alpha-adducin G460W genotype, men and women aged 30-74 years.

	GG	GW	WW	p for difference of GG vs WW
Men	237	505	306	
Systolic blood pressure, mmHg	133.1	134.8	135.4	0.14
Diastolic blood pressure, mmHg	80.8	80.7	81.2	0.89
Women	362	860	553	
Systolic blood pressure, mmHg	131.4	131.4	129.8	0.15
Diastolic blood pressure, mmHg	77.1	77.1	76.6	0.60
Total	599	1,365	859	
Systolic blood pressure, mmHg	132.0	132.7	131.8	0.95
Diastolic blood pressure, mmHg	78.5	78.5	78.3	0.87
Men ages <55 years	80	176	90	
Systolic blood pressure, mmHg	131.1	127.9	129.6	0.68
Diastolic blood pressure, mmHg	83.3	80.5	82.5	0.82
Men ages ≥55 years	157	329	216	
Systolic blood pressure, mmHg	134.0	138.3	138.2	0.02
Diastolic blood pressure, mmHg	79.4	80.9	80.6	0.38

Blood pressures were adjusted for sex, age, antihypertensive medication use, body mass index and alcohol consumption.
Tests for difference from GG genotype were conducted using Dunnett's multiple comparison method.

Table 3. Blood pressure levels according to alpha-adducin G460W genotype, stratified by sodium excretion/intake, men aged 30-74 years.

	Men			
	GG	**GW**	**WW**	p for difference of GG vs WW
Stratified by urinary sodium excretion				
<189.4 mmol/day	85	161	92	
Systolic blood pressure, mmHg	133.9	137.9	134.2	0.99
Diastolic blood pressure, mmHg	79.9	81.5	81.3	0.51
≥189.4 mmol/day	80	159	99	
Systolic blood pressure, mmHg	133.6	134.6	138.8	0.02
Diastolic blood pressure, mmHg	79.9	80.5	81.5	0.41
Stratified by present sodium intake score				
<6	97	208	144	
Systolic blood pressure, mmHg	136.1	136.3	138.6	0.31
Diastolic blood pressure, mmHg	81.8	82.0	82.2	0.91
≥6	138	292	159	
Systolic blood pressure, mmHg	130.7	133.8	132.3	0.53
Diastolic blood pressure, mmHg	79.9	79.9	80.3	0.93
Stratified by past sodium intake score				
<8	135	304	191	
Systolic blood pressure, mmHg	132.9	134.9	134.8	0.42
Diastolic blood pressure, mmHg	80.3	80.9	82.0	0.20
≥8	98	192	111	
Systolic blood pressure, mmHg	132.2	135.0	136.3	0.08
Diastolic blood pressure, mmHg	80.9	80.6	79.8	0.59

Blood pressures were adjusted for sex, age, antihypertensive medication use, body mass index and alcohol consumption.
Tests for difference from GG genotype were conducted using Dunnett's multiple comparison method.