Associations Between Genetic Variants of NADPH Oxidase-Related Genes and Blood Pressure Responses to Dietary Sodium Intervention: The GenSalt Study

Xikun Han,[1] Zunsong Hu,[1] Jing Chen,[2] Jianfeng Huang,[1] Chen Huang,[1] Fangchao Liu,[1] Charles Gu,[3] Xueli Yang,[1] James E. Hixson,[4] Xiangfeng Lu,[1] Laiyuan Wang,[1] De-Pei Liu,[5] Jiang He,[2] Shufeng Chen,[1] and Dongfeng Gu[1]

BACKGROUND
The aim of this study was to comprehensively test the associations of genetic variants of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-related genes with blood pressure (BP) responses to dietary sodium intervention in a Chinese population.

METHODS
We conducted a 7-day low-sodium intervention followed by a 7-day high-sodium intervention among 1,906 participants in rural China. BP measurements were obtained at baseline and each dietary intervention using a random-zero sphygmomanometer. Linear mixed-effect models were used to assess the additive associations of 63 tag single-nucleotide polymorphisms in 11 NADPH oxidase-related genes with BP responses to dietary sodium intervention. Gene-based analyses were conducted using the truncated product method. The Bonferroni method was used to adjust for multiple testing in all analyses.

RESULTS
Systolic BP (SBP) response to high-sodium intervention significantly decreased with the number of minor T allele of marker rs6967221 in *RAC1* ($P = 4.51 \times 10^{-4}$). SBP responses (95% confidence interval) for genotypes CC, CT, and TT were 5.03 (4.71, 5.36), 4.20 (3.54, 4.85), and 0.56 (−1.08, 2.20) mm Hg, respectively, during the high-sodium intervention. Gene-based analyses revealed that *RAC1* was significantly associated with SBP response to high-sodium intervention ($P = 1.00 \times 10^{-6}$) and diastolic BP response to low-sodium intervention ($P = 9.80 \times 10^{-4}$).

CONCLUSIONS
These findings suggested that genetic variants of NADPH oxidase-related genes may contribute to the variation of BP responses to sodium intervention in Chinese population. Further replication of these findings is warranted.

Keywords: blood pressure; genetic association; hypertension; NADPH oxidase; salt sensitivity.

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Essential hypertension is a major risk factor of cardiovascular disease and has become a serious public health problem.[1] An excess of dietary salt is one of the established environmental factors of hypertension.[2,3] Blood pressure (BP) response to dietary sodium intake varies considerably among individuals, termed salt sensitivity of BP (SSBP).[4] The Genetic Epidemiology Network of Salt-Sensitivity (GenSalt) study indicates that the heritability of SSBP ranges from 20% to 33% among people from northern rural area in China.[3] However, the genomic mechanisms of SSBP remain to be elucidated.

Reactive oxygen species, including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl anion (OH^-), play an important role in hypertension.[5,6] Increased renal and vascular oxidative stress is implicated in SSBP.[7] Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (Noxs) are major sources to increase the level of reactive oxygen species.[8] NADPH oxidase is a multicomponent enzyme consisting of at least one membrane-bound NOX subunit. The classical NADPH oxidase is comprised of 2 integral membrane proteins, the catalytic subunits gp91phox (now referred to as *NOX2*) and p22phox (*CYBA*),

Correspondence: Dongfeng Gu (gudongfeng@vip.sina.com).

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[1]Department of Epidemiology, State Key Laboratory of Cardiovascular Disease, Fuwai Hospital, National Center for Cardiovascular Diseases, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China; [2]Department of Epidemiology, Tulane University School of Public Health and Tropical Medicine, New Orleans, Los Angeles, USA; [3]School of Medicine, Washington University, St. Louis, Missouri, USA; [4]School of Public Health, University of Texas, Houston, Texas, USA; [5]National Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China.

and the cytosolic components p47phox (*NCF1*), p67phox (*NCF2*), p40phox (*NCF4*), and Rac1 (*RAC1*) or 2 (*RAC2*).[8] In mammalian, 7 distinct NOX genes (*NOX1* to 5 and *DUOX1* and 2) have been identified.[9] Among them, only *NOX1*, *NOX2*, *NOX4*, and *NOX5* have been identified in the cardiovascular–renal systems and have been implicated in the pathophysiology of cardiovascular and renal disease.[10,11]

Recently, evidence from Dahl salt-sensitive (SS) rats revealed that *NOX4*, *NCF2*, and *RAC1* contributed to the development of salt-induced hypertension.[12–14] For instance, $SS^{Nox4-/-}$ rats had reduction of renal injury and attenuation of BP response to high salt compared with SS rats.[12] In comparison to a salt-resistant strain, the higher expression of *NCF2* was associated with higher NADPH oxidase activity and salt sensitivity in SS rats.[13] In Dahl SS rats, high-salt status activates renal *RAC1* in SS hypertension, leading to high BP and renal damage.[14] Previous candidate gene studies have reported associations between common variants of the *CYBA* gene and BP, hypertension, and SSBP.[15–17] However, the comprehensive relationship of NADPH oxidase-related genes polymorphisms with SSBP had not been investigated. Thus, we systematically selected 11 NADPH oxidase-related genes (Table 1) and conducted single-marker and gene-based analyses to examine the associations of 11 NADPH oxidase-related genes with BP response to sodium intervention among 1,906 Chinese participants in the GenSalt study.

METHODS

Study population

The GenSalt study was conducted in a Han Chinese population from rural areas in northern China. A community-based BP screening was carried out among residents aged 18–60 years in the study villages to identify potential probands and their families. Those with a mean systolic BP (SBP) between 130–160 mm Hg and/or a diastolic BP (DBP) between 85–100 mm Hg and no use of antihypertensive medications as well as their spouses, siblings, and offspring were recruited as volunteers for the dietary intervention study. Individuals with stage 2 hypertension, current use of antihypertensive medications, secondary hypertension, history of clinical cardiovascular disease, diabetes, chronic kidney disease, along with pregnant women, heavy alcohol users, and those currently on a low-sodium diet were excluded from the study. The detailed eligibility criteria for the probands and their family members are presented elsewhere.[18] Institutional Review Boards at all of the participating institutions approved the GenSalt study. Written informed consents for the baseline observation and for the intervention program were obtained from each participant.

Dietary intervention

The study participants received a 7-day low-sodium intervention (3 g of sodium chloride or 51.3 mmol of sodium per day) followed by a 7-day high-sodium intervention (18 g of sodium chloride or 307.8 mmol of sodium per day). Total energy intake was varied according to each participant's baseline energy intake. All of the foods were cooked without salt, and prepackaged salt was added to the individual study participant's meal when it was served by the study staff. All participants were required to have their breakfast, lunch, and dinner at the study kitchen under supervision of the study staff during the entire study period and they were instructed to avoid consuming any foods that were not provided by the study. To ensure study participants' compliance to the dietary sodium intervention, 3 timed urinary specimens (one 24-hour and 2 overnight) were collected at baseline and at the end of each phase of intervention (days 5, 6, and 7). The overnight urinary excretion of sodium was converted to 24-hour values on the basis of formula developed from a random sample of 238 participants.[19] The mean (SD) 24-hour urinary excretions of sodium was 242.4 (66.7) mmol at baseline, 47.5 (16.0) mmol during the low-sodium intervention, and 244.3 (37.7) mmol during the high-sodium intervention, respectively, which showed excellent compliance with the study diet. Among the 1,906 eligible participants, 1,871

Table 1. Characteristics of 11 NADPH oxidase-related genes

	Physical position			
Gene symbol	Chr	±5 kb	Tag SNPs	Encoded protein
NCF2	1	(183519697, 183565056)	5	Neutrophil cytosolic factor 2, p67phox
RAC1	7	(6409126, 6448598)	14	RAS-related C3 botulinum toxin substrate 1
NOXA1	9	(140312847, 140333858)	1	NADPH oxidase activator 1
NOX4	11	(89052522, 89229653)	17	NADPH oxidase 4
NOX5	15	(69302034, 69354501)	3	NADPH oxidase 5
CYBA	16	(88704697, 88722492)	1	Cytochrome b-245, alpha polypeptide, p22phox
NOXO1	16	(2023918, 2036550)	3	NADPH oxidase organizer 1
NCF4	22	(37252030, 37279059)	5	Neutrophil cytosolic factor 4, p40phox
RAC2	22	(37616310, 37645305)	9	RAS-related C3 botulinum toxin substrate 2
NOX1	X	(100093313, 100134334)	2	NADPH oxidase 1
NOX2	X	(37634270, 37677714)	3	NADPH oxidase 2, p91phox

Abbreviations: NADPH, nicotinamide adenine dinucleotide phosphate; SNP, single-nucleotide polymorphism.

(98.2%) and 1,860 (97.6%) completed the low-sodium and high-sodium interventions, respectively, and were included in the current analysis.

Phenotype measurements

During the 3 days of baseline examination, trained staff collected information on family structure, demographic characteristics, personal and family medical history, and lifestyle risk factors using a standard questionnaire. Three morning BP measurements were obtained according to a standard protocol during each day of baseline observation and on days 5, 6, and 7 of each intervention period. All BP readings were measured by trained and certified observers using random-zero sphygmomanometer.[20] BP was measured with the participants in the sitting position after 5 minutes of rest. In addition, participants were advised to avoid alcohol, cigarette smoking, coffee/tea, and exercise for at least 30 minutes prior to their BP measurements. In addition, body weight, height, and waist circumference were measured twice in light indoor clothing without shoes during the baseline examination. Body mass index (BMI) was calculated as kilograms per meters squared (kg/m^2).

BP responses were defined continuously as the absolute changes in SBP, DBP, and mean arterial pressure when switching from baseline to low-sodium intervention and from low-sodium to high-sodium intervention. Mean BP responses to low-sodium intake were calculated as the mean of 9 measurements on days 5, 6, and 7 during the low-sodium intervention minus the mean of 9 measurements at baseline, and responses to high-sodium intake were calculated as the mean of 9 measurements on days 5, 6, and 7 during the high-sodium intervention minus the mean of 9 measurements on days 5, 6, and 7 during the low-sodium intervention.

Genotype data and quality control

Eleven NADPH oxidase-related genes were selected based on their potential biological effects on BP regulation (Table 1). Within the 11 candidate genes, 124 single-nucleotide polymorphisms (SNPs) were genotyped on the Affymetrix 6.0 platform (Affymetrix, Santa Clara, CA). Quality control excluded 26 SNPs based on low minor allele frequency <1%, low genotyping call rate (<95%), or deviation from the Hardy–Weinberg equilibrium after using the false discovery rate procedure (Q value with the significance level of 0.05 was used) to correct for multiple testing. Among the remaining 98 SNPs, 63 tag SNPs were selected using Haploview software (version 4.2, http://www.broad.mit.edu/mpg/haploview) with r^2 <0.8 for inclusion in the statistical analysis.[21] Quality control information on the tagged 63 SNPs was listed in Supplementary Table 1.

Statistical analysis

Quality control, including checks of Mendelian consistency, genotyping call rate, minor allele frequency, and Hardy–Weinberg equilibrium was performed using PLINK software (version 1.07; Dr Sean Purcell, http://pngu.mgh.harvard.edu/~purcell/plink/).[22]

The means or percentages of phenotypic and genotypic data were calculated for the 1,906 GenSalt study participants. Additive associations between SNPs and BP responses to each dietary sodium intervention were assessed by a mixed-effect linear regression model using the PROC MIXED procedure in SAS (version 9.3; SAS Institute, Cary, NC).[23] The mixed-effect model we used was as follows:

$$\gamma_{ij} = \beta_0 + \beta_1 \times age_{ij} + \beta 2 \times gender_{ij} + \beta_3 \times BMI_{ij} + \beta_4 \times 24\_hour\_urinary\_sodium_{ij} + \beta_5 \times SNP_{ij} + a_j + e_{ij}$$

In the formula, γ_{ij} represents the BP responses to each dietary sodium intervention for the ith individual in the jth family. β_0 was the mean BP responses after accounting for covariates and genetic effects. The terms age_{ij}, $gender_{ij}$, BMI_{ij}, and $24\_hour\_urinary\_sodium_{ij}$ represented baseline age, gender, BMI, and 24-hour urinary sodium excretion of the ith individual in the jth family, respectively. SNP_{ij} modeled the genetic effect, where the genotype was coded under an additive model. The random effects term a_j accounts for the correlation among individuals in the same family. The last term e_{ij} stands for residual. P values for β_5 were used to evaluate the significance of the association of each SNP with BP responses to each dietary sodium intervention.

A sandwich estimator was used to account for the nonindependence of family members. To account for the sex-specific structure genes in X chromosome, models were calculated that assumed inactivation as well as not assuming inactivation. A similar, gender-stratified analysis was conducted for those SNPs located on the X-chromosome. For significant SNPs, the mean effect size and 95% confidence interval was estimated for each genotype using a mixed-linear regression model. Comparatively, to evaluate our results from the mixed-effect models, we also used the packages kinship2 and GWAF to account for the kinships between individuals in R software (version 3.2.4; http://www.r-project.org).

In the gene-based analysis, the truncated product method (TPM) was used to determine the overall association of each NADPH oxidase-related genes, in which at least 2 SNPs were genotyped, with BP responses to dietary sodium intervention.[24] The truncation point was set as $\tau = 0.10$, and the P value for TPM was estimated by 1,000,000 simulations. Sensitivity analyses were conducted using the TPM after removing the lead SNP within a gene to examine their influence on the gene-based association. Additionally, to evaluate the robustness of findings from the TPM, the Versatile Gene-based Association Study (VEGAS) was also employed.[25,26]

Bonferroni correction was used to adjust for multiple comparisons. The thresholds for the single SNP-based and gene-based analyses were $\alpha = 0.05/63 = 7.94 \times 10^{-4}$ and $\alpha = 0.05/9 = 5.56 \times 10^{-3}$, respectively. Single-SNP-based analysis was conducted using SAS (version 9.3; SAS Institute). TPM was performed using R software (version 3.2.4; http://www.r-project.org).

RESULTS

Characteristics of the GenSalt participants and BP responses to sodium interventions were shown in Table 2.

Table 2. Characteristics of 1,906 GenSalt dietary intervention participants

Variable	Mean ± SD or percentage	Median (interquartile range)
Age, years	38.7 ± 9.6	39.0 (33.0, 46.0)
Men, %	53.0	
BMI, kg/m^2	23.3 ± 3.2	22.9 (21.1, 25.2)
SBP, mm Hg		
Baseline	116.9 ± 14.2	115.8 (106.4, 127.1)
Response to low-sodium intake	−5.5 ± 7.0*	−4.4 (−8.9, −1.3)
Response to high-sodium intake	4.9 ± 6.0*	4.7 (0.6, 8.2)
DBP, mm Hg		
Baseline	73.7 ± 10.3	73.3 (66.7, 80.7)
Response to low-sodium intake	−2.8 ± 5.5*	−2.7 (−5.6, 0.4)
Response to high-sodium intake	1.9 ± 5.4*	1.8 (−1.6, 5.3)
MAP, mm Hg		
Baseline	88.1 ± 10.9	87.7 (80.0, 95.4)
Response to low-sodium intake	−3.7 ± 5.3*	−3.3 (−6.6, −0.6)
Response to high-sodium intake	2.9 ± 5.0*	2.7 (−0.4, 5.9)

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; MAP, mean arterial pressure; SBP, systolic blood pressure. *P value <0.0001 when compared to no BP change during sodium interventions.

On average, study participants were 38.7 years old and had a mean BMI of 23.3 kg/m^2, mean SBP of 116.9 mm Hg, and mean DBP of 73.7 mm Hg. Approximately 53% of participants were male. BP levels were similar during baseline and the high-sodium intervention, and BP responses to sodium intervention were significantly different from zero.

Figure 1 presents the association of each SNP with absolute SBP, DBP, and mean arterial pressure responses to sodium intervention. After adjustment for multiple testing, *RAC1* marker rs6967221 was significantly associated with SBP response to high-sodium intervention ($P = 4.51 \times 10^{-4}$). Mean SBP responses (95% confidence interval) were 5.03 (4.71, 5.36), 4.20 (3.54, 4.85), and 0.56 (−1.08, 2.20) mm Hg for genotypes CC, CT, and TT, respectively, during the high-sodium intervention (Table 3). Exact P values for all SNPs association tests were shown in Supplementary Table 2. Similar results were obtained after including kinships between individuals.

Table 4 presents the results of gene-based analyses. *CYBA* and *NOXA1* could not be examined since only one SNP was available within each of these genes. Among the remaining 9 genes, *RAC1* was significantly associated with DBP response to low-sodium intervention ($P = 9.80 \times 10^{-4}$) and SBP response to high-sodium intervention ($P = 1.00 \times 10^{-6}$) even after adjustment for multiple testing. In sensitivity analyses, the overall associaitons of *RAC1* with SBP response to high-sodium intervention ($P = 6.20 \times 10^{-5}$) remained significant even after removing the lead marker rs6967221 from gene-based analyses. DBP responses to low-sodium intervention ($P = 0.02$) remained nominal significant after the removal of the lead SNP rs2689420. *NOX4* showed nominal significance with DBP response to low-sodium intervention ($P = 0.01$). Similar results were obtained using the VEGAS analyses (Supplementary Table 3).

DISCUSSION

In the present study, we examined the association between the NADPH oxidase-related genes and SSBP among a large sample of Han Chinese population. We identified that the minor T allele of rs6967221 in *RAC1* was associated with the decreased SBP response to high-sodium intervention. Consistent with these findings, gene-based analyses also revealed a significant association of the *RAC1* gene with DBP response to low-sodium intervention and SBP response to high-sodium intervention. Besides, *NOX4* was nominally associated with DBP response to low-sodium intervention. In aggregate, these findings contribute genetic evidence for a role of NADPH oxidase-related genes in SSBP.

Rac1 is a member of the Rho family of GTPases, and it cycles between an inactive GDP-bound and an active GTP-bound state.[27] It is part of the NADPH oxidase complex, which induces the generation of reactive oxygen species leading to increased oxidative stress, alterations in the cell membrane, and endothelial damage.[28] Several studies have provided important evidence of the link between *RAC1* and BP control and SSBP.[29–31] In Dahl SS rats, it is reported that enhanced NaCl–Rac1–NADPH oxidase–reactive oxygen species–Na reabsorption cascade might be an important mechanism of SS hypertension.[32] Besides, several studies reported that Rac1-mineralocorticoid receptor pathway plays a key role in the development of SS hypertension.[14,33,34] However, few studies have implicated the association between *RAC1* gene variants and SSBP. To the best of our knowledge, this is the first investigation to examine the association of genetic variants in *RAC1* with SSBP. The marker rs6967221 locates in the intronic region of the *RAC1* gene. Although we used the web tools SNPinfo and RegulomeDB to speculate the functional implication of the significant SNP,[35,36] little evidence showed that rs6967221 and its highly correlated SNPs are causally

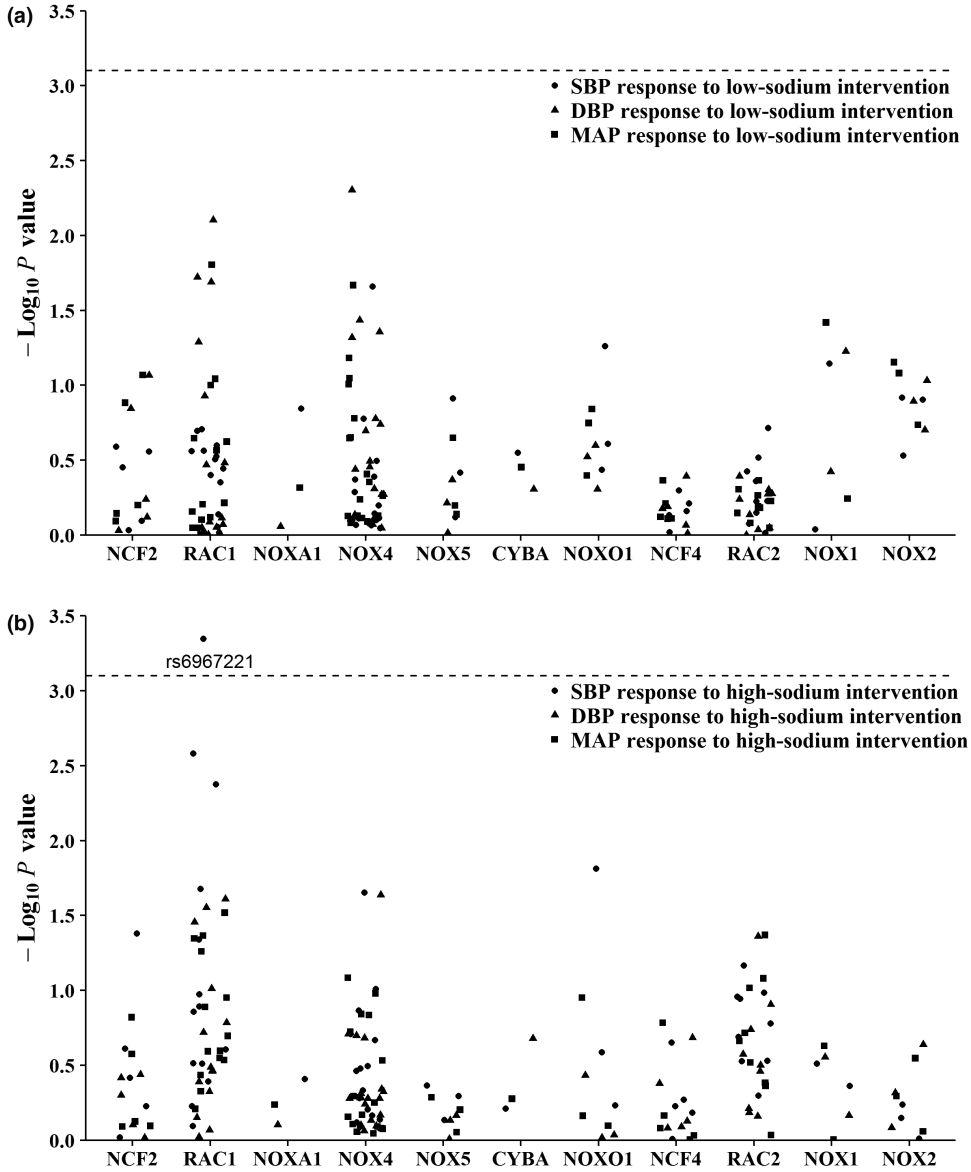

Figure 1. -Log_{10} *P* values for the associations of 63 tag SNPs in NADPH oxidase-related genes with blood pressure responses to low-sodium (**a**) and high-sodium (**b**) interventions (points were jittered to reduce overlap). Labeled SNP was significant after Bonferroni correction. The horizontal dashed lines indicated the Bonferroni corrected significant level ($P = 7.94 \times 10^{-4}$). Abbreviations: NADPH, nicotinamide adenine dinucleotide phosphate; SNP, single-nucleotide polymorphism.

associated with the regulation of *RAC1* expression. Thus, the function of rs6967221 needs to be further investigated. A previous study in a Chilean pediatric population demonstrated that polymorphism rs836478 and rs10951982 of the *RAC1* gene was associated with hypertension and DBP.[37] SNP rs10951982 was not genotyped in our study. Due to weak correlation between rs10951982 and rs6967221 ($r^2 = 0.012$) in HapMap Chinese Han in Beijing data, rs6967221 could be a novel marker associated with BP response to diet salt intervention in Chinese. Besides, the present study showed that rs836478 was nominally associated with DBP and mean arterial pressure responses to low-sodium (*P* values = 0.001 and 0.043, respectively) and high-sodium interventions (*P* values = 0.014 and 0.023, respectively). Furthermore, finding from gene-based analysis supported that *RAC1* influenced the BP responses to dietary sodium intake even after removing the prominent marker rs6967221. Further genetic and functional research are still needed to delineate the role of *RAC1* in SSBP.

Several studies indicated that *CYBA* variants influenced the indices of oxidative stress and were associated with hypertension and SSBP.[15–17,38] For instance, the *CYBA* gene C242T (rs4673) polymorphism was associated with SSBP in Hispanics.[16] With only rs12709102 available in the current study, we could not replicate these findings. Several possibilities may explain the discrepancies among studies. Our study was conducted in Han Chinese population, whose linkage disequilibrium structure may be different from other populations. Furthermore, the same genetic variants may have different BP effects considering gene–environment interaction.

Table 3. BP response to dietary sodium intervention according to rs6967221 genotype

		Low-sodium intervention		High-sodium intervention	
BP response	Genotype	Absolute change (95% CI)	*P* value for additive model	Absolute change (95% CI)	*P* value for additive model
SBP	CC	−5.60 (−5.97, −5.22)	0.27	5.03 (4.71, 5.36)	4.51×10^{-4}
	CT	−5.07 (−5.85, −4.29)		4.20 (3.54, 4.85)	
	TT	−5.85 (−7.78, −3.92)		0.56 (−1.08, 2.20)	
DBP	CC	−2.81 (−3.12, −2.50)	0.89	2.00 (1.68, 2.32)	0.41
	CT	−2.93 (−3.63, −2.24)		1.85 (1.21, 2.49)	
	TT	−2.27 (−4.92, 0.38)		0.60 (−2.11, 3.32)	
MAP	CC	−3.74 (−4.03, −3.44)	0.75	3.01 (2.72, 3.30)	5.50×10^{-2}
	CT	−3.65 (−4.31, −2.99)		2.64 (2.07, 3.21)	
	TT	−3.46 (−5.59, −1.34)		0.60 (−1.50, 2.69)	

Abbreviations: CI, confidence interval; DBP, diastolic blood pressure; MAP, mean arterial pressure; SBP, systolic blood pressure.

Table 4. Associations of 9 NADPH oxidase-related genes with BP response to dietary sodium intervention (truncated product method)

Gene[a]		Absolute blood pressure response					
		Low-sodium intervention			High-sodium intervention		
	Number of SNPs	SBP	DBP	MAP	SBP	DBP	MAP
NCF2	5	0.39	0.40	0.37	0.22	0.37	0.37
RAC1	14	0.67	9.80×10^{-4}[b]	0.09	1.00×10^{-6}[b]	0.01	0.12
NOX4	17	0.44	0.01	0.13	0.32	0.37	0.66
NOX5	3	0.26	0.28	0.22	0.17	0.25	0.20
NOXO1	3	0.13	0.15	0.17	0.05	0.29	0.29
NCF4	5	0.28	0.33	0.20	0.44	0.32	0.46
RAC2	9	0.47	0.60	0.26	0.21	0.28	0.09
NOX1	2	0.14	0.11	0.08	0.12	0.17	0.20
NOX2	3	0.16	0.13	0.09	0.26	0.24	0.25

Abbreviations: DBP, diastolic blood pressure; MAP, mean arterial pressure; NADPH, nicotinamide adenine dinucleotide phosphate; SBP, systolic blood pressure; SNP, single-nucleotide polymorphism.

[a]Genes containing at least 2 SNPs.

[b]Significant after adjustment for multiple testing using Bonferroni correction.

Moreover, other potential factors, such as different characteristics of subjects, sodium intervention design, and lifestyle factors, might contribute to the inconsistency of results.

Strengths and limitations

Several strengths of this study should be noted. First of all, it is a comprehensive investigation to examine associations of NADPH oxidase-related genes with BP responses to dietary sodium intervention. Study attributes, such as homogeneity of the population, should make the analysis robust to population stratification. In addition, measurement of BP for 9 times during each period had ensured the accuracy of BP and stringent quality control procedures were also conducted for genotyping data, data collection, and dietary intervention. Moreover, Bonferroni correction procedures were performed accounting for multiple testing. However, this study also had several limitations. Our research was conducted in a Han Chinese population. These novel associations reported here need be replicated in other populations with different genetic background. Thus, the generalizability of our results to other populations with different genetic and environmental background was unknown. Furthermore, although the Affymetrix 6.0 platform generally provides good genomic coverage of common polymorphisms in the Han Chinese population (approximately 75%),[39] limited genotype data were available for the *CYBA* and *NOXA1* genes. Therefore, future researches to examine the associations between common variants in these genes and SSBP are still needed.

In conclusion, our study suggested that common variants of NADPH oxidase-related genes were associated with BP responses to dietary sodium intervention in Han Chinese. These findings may contribute to a better understanding of the genetic mechanism of underlying BP regulation.

However, replications of these results in other populations with different genetic background are needed. Furthermore, functional studies are warranted to pinpoint the underlying mechanism.

SUPPLEMENTARY MATERIAL

Supplementary data are available at *American Journal of Hypertension* online.

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DISCLOSURE

The authors declared no conflict of interest.

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