Journal of Human Hypertension (2018) 32:287–293
https://doi.org/10.1038/s41371-018-0041-6

ARTICLE

Associations of NADPH oxidase-related genes with blood pressure changes and incident hypertension: The GenSalt Study

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Received: 9 July 2017 / Revised: 3 January 2018 / Accepted: 22 January 2018 / Published online: 20 February 2018

Abstract

Previous studies have indicated that reactive oxygen species produced by NADPH oxidase (Nox) are important risk factors of hypertension. The current study aims to examine the associations of Nox-related genes with longitudinal blood pressure (BP) changes and the risk of incident hypertension in the Genetic Epidemiology Network of Salt Sensitivity (GenSalt) follow-up study. A total of 1,768 participants from 633 families were included in our analysis. Nine BP measurements were obtained in the morning at baseline and during two follow-up visits. The mixed-effect models were used to investigate the associations of 52 tagged single-nucleotide polymorphisms in 11 Nox-related genes with BP changes and incident hypertension. Gene-based analyses were performed by truncated product method (TPM) and Versatile Gene-based Association Study (VEGAS). Over the 7.2 years of follow-up, systolic BP (SBP) and diastolic BP (DBP) increased, and 32.1% (512) of participants developed hypertension. SNPs rs12094228, rs16861188 and rs12066019 in *NCF2* were significantly associated with longitudinal change in SBP ($P_{interaction} = 1.1 \times 10^{-3}$, 2.8×10^{-3} and 1.2×10^{-3}, respectively). Gene-based analyses revealed that *NCF2* was significantly associated with SBP ($P_{TPM} = 1.00 \times 10^{-6}$, $P_{VEGAS} = 1.26 \times 10^{-4}$) and DBP changes ($P_{TPM} = 5.84 \times 10^{-4}$, $P_{VEGAS} = 1.04 \times 10^{-3}$). These findings suggested that *NCF2* may play an important role in BP changes over time in the Han Chinese population.

Introduction

Elevated blood pressure (BP) is the leading modifiable risk factor for cardiovascular diseases and global burden of disease worldwide [1, 2]. BP is also a classical complex genetic trait with an estimated heritability of 31–68% [3]. Although genome-wide association studies (GWAS) have identified over 120 genetic regions associated with BP [4, 5], the exact genomic mechanism underlying BP regulation remains to be clarified.

Reactive oxygen species (ROS), including superoxide anion ($O2^-$), hydrogen peroxide (H_2O_2), and hydroxyl anion (OH^-), play an important role in the pathogenesis of hypertension, as they could affect nitric oxide (NO) bioavailability, peroxynitrite ($ONOO^-$) generation and redox senstive signaling pathyways [6]. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (Noxs) are major sources of ROS in cardiovascular and renal systems [7, 8]. The classical Nox is a multicomponent enzyme comprised of the cytosolic subunits p40phox (*NCF4*), p47phox (*NCF1*), p67phox (*NCF2*), and Rac1 (*RAC1*) or 2 (*RAC2*), and the catalytic subunits p22phox (*CYBA*) and

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Electronic supplementary material The online version of this article (https://doi.org/10.1038/s41371-018-0041-6) contains supplementary material, which is available to authorized users.

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gp91phox (now referred to as *NOX2*) [8]. In mammalian, there are seven distinct NOX genes (*NOX1–5* and *DUOX1* and *2*) [9], and only *NOX1*, *NOX2*, *NOX4*, and *NOX5* are identified in cardiovascular renal systems and play an important role in renal and cardiovascular disease [7, 10].

Extensive experimental data support the role of Noxs in BP regulation and pathogenesis of hypertension. In Ang II-infused rats and mice, expression and activity of Noxs, ROS generation and BP were increased [11, 12]. In p47phox knockout mice, Ang II infusion failed to increase vascular O_2^- production and hypertensive response was markedly blunted [13]. In population studies, hypertensive patients have higher levels of plasma H_2O_2 and oxidative stress than normotensive individuals [6]. Common genetic variants of *NOX4*, *CYBA* and *RAC1* have been reported to be associated with BP levels and hypertension [4–6, 14, 15]. However, few studys have investigated the association of Nox-related genes polymorphisms with BP changes and the risk of incident hypertension. In the current study we systematically selected 11 Nox-related genes (*NCF2*, *RAC1*, *NOXA1*, *NOX4*, *NOX5*, *NOXO1, CYBA*, *NCF4*, *RAC2*, *NOX2*, and *NOX1*) and conducted single-marker and gene-based analyses to examine the associations of these genes with BP changes and hypertension among participants in the Genetic Epidemiology Network of Salt Sensitivity (GenSalt) study.

Materials and methods

Study population

The GenSalt study was conducted in a Han Chinese population from rural areas in northern China. The detailed information of the design and methods has been presented elsewhere [16]. Briefly, we used BP screening to identify potential probands among persons aged 18–60 years. Those with systolic BP (SBP) 130–160 mm Hg and/or diastolic BP (DBP) 85–100 mm Hg and no current or recent (less than 1 month before screening visit) use of antihypertensive medications as well as their offspring, siblings, spouses and parents were recruited in GenSalt 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 GenSalt study was approved by Institutional Review Boards at all of the participating institutions in accordance with the Declaration of Helsinki. Written informed consents for the program were obtained from each participant.

Baseline data collection

The baseline examination was performed from 2003 to 2005. During the examination, information about family pedigrees, demographic characteristics, personal and family medical history, and lifestyle risk factors was obtained using a standard questionnaire which was administered by trained staff. In the morning of 3-day baseline observation period, nine BP measurements were obtained using a random-zero sphygmomanometer by trained technicians based on a standard protocol. Besides, before BP measurements, participants were advised to avoid coffee/tea, cigarette smoking, alcohol, and exercise. Mean BP was the average of nine BP measurements in the baseline period.

Follow-up data collection

There were two follow-up examinations for GenSalt participants in 2008–2009 and 2011–2012. During each follow-up visit, a 3-day examination was performed as that of the baseline period. Information on the history of hypertension and use of antihypertension medications was obtained using a standard questionnaire. Three BP measurements were obtained using a random-zero sphygmomanometer in the morning during each of 3 days of follow-up visits. The mean of the nine BP measurements was calculated for the current analysis. Hypertension was defined as having a SBP ≥ 140 mm Hg or DBP ≥ 90 mm Hg or use of antihypertensive medications.

Among 1906 eligible individuals who participated in the GenSalt baseline examination, 117 individuals without BP data at both follow-up visits and another 21 individuals without genotype data were excluded. The remainning 1768 participants (92.8%) were analyzed in the current study.

Genotype data and quality control

A total of 11 Nox-related genes were selected and the detailed information of the genes has been presented elsewhere [15]. Among 124 SNPs genotyped on the Affymetrix 6.0 platform (Affymetrix, Santa Clara, CA), 26 SNPs with low genotyping call rate (<95%), low minor allele frequency (MAF) <1%, or deviation from the Hardy–Weinberg equilibrium (HWE) were excluded. For the remaining 98 SNPs, we used Haploview software (version 4.2, http://www.broad.mit.edu/mpg/haploview) to select tag SNPs with $r^2 < 0.8$ [17]. Supplementary Table 1 presented the information of the final 52 tagged SNPs.

Statistical analysis

Quality control, including genotyping call rate, Mendelian consistency, MAF and HWE, was conducted by PLINK software (http://zzz.bwh.harvard.edu/plink/) [18].

Descriptive statistics were shown for the 1,768 participants. The additive association between SNPs and BP changes over time were assessed by mixed-effect linear regression models to account for the longitudinal, family-based design of the GenSalt study. Autoregressive and compound symmetry covariance matrices were used to account for the correlations of repeated measurements within individuals and of individuals within families, respectively. For the assessment of BP changes over time, the main effects of these variables and a genotype by follow-up time interaction term were included in the models. Briefly, genotype, follow-up time, a genotype by follow-up time interaction term, the fixed effects of baseline age, gender, and body mass index (BMI) were included in models using the PROC MIXED procedure in SAS (version 9.3; SAS Institute, Cary, NC) [19]. To account for the effects of antihypertensive medication, we conducted these analyses using imputed BP levels for participants taking antihypertensive medication by adding 10 and 5 mm Hg to original SBP and DBP values, respectively [20]. A sensitivity analysis was also conducted after excluding those participants taking antihypertensive medicine in the month prior to follow-up visit.

For examination of incident hypertension, 173 participants with hypertension at baseline were excluded. The additive associations between SNPs and incident hypertension were employed using generalized linear mixed models [21]. Autoregressive and compound symmetry covariance matrices were once again used to account for the correlations of repeated measurements intraindividual and interindividuals within families, respectively. The baseline age, gender, BMI, and follow-up time were adjusted in the multivariable analysis by the PROC GLIMMIX procedure in SAS.

To validate our results from the mixed-effect models, we also used the R packages "kinship2" and "GWAF" to account for the genetic kinships among individuals (http://www.r-project.org). To account for the sex-specific structure genes (*NOX1* and *2*) on X chromosome, the models were assumed inactivation as well as not assuming inactivation. Gender-stratified analysis was also conducted for those SNPs located at the X chromosome.

Truncated product method (TPM) was performed for gene-based analysis of each Nox-related genes (at least two SNPs were genotyped) with longitudinal BP changes and incident hypertension [22]. In TPM, the *P*-values were estimated by 1,000,000 simulations with the truncation point as 0.10. We also employed the Versatile Gene-based Association Study (VEGAS) to evaluate the robustness of findings from the TPM [23, 24]. The analysis was performed using the command line tool of VEGAS2 version 1 [24].

The false discovery rate method was used to adjust for multiple testing [25]. More specifically, we used the PROC MULTTEST procedure, along with the false discovery rate option in SAS to calculate the *Q*-values for single SNP-based and gene-based analyses. *Q*-values <0.05 were considered statistically significant.

Table 1 Characteristics of 1,768 GenSalt follow-up study participants

	Baseline ($N = 1768$)	Visit 1 ($N = 1687$)	Visit 2 ($N = 1626$)
Age, years	39.0 ± 9.2	43.9 ± 9.2	46.7 ± 9.2
Men, N(%)	924 (52.3)	878 (52.0)	845 (52.0)
BMI, kg/m^2	23.4 ± 3.2	24.1 ± 3.4	24.8 ± 3.5
SBP, mmHg	116.9 ± 14.1	122.4 ± 16.8	129.1 ± 17.3
DBP, mmHg	73.8 ± 10.2	78.9 ± 11.0	82.2 ± 11.1
Hypertension at baseline, *N* (%)	173 (9.8)	–	–
Hypertension incidence, *N* (%)[a]	–	264 (16.6)	512 (32.1)
Follow-up time, years	–	4.6 ± 0.7	7.2 ± 0.9

Data are presented as mean ± SD or percentages

BMI body mass index, *DBP* diastolic blood pressure, *N* number of participants, *SBP* systolic blood pressure

[a] Participants with hypertension at baseline were excluded

RESULTS

Table 1 shows the characteristics of the 1,768 participants at baseline and two follow-up interviews. On average, study participants were 39.0 years old and had a BMI of 23.4 kg/m^2, mean SBP of 116.9 mm Hg and mean DBP of 73.8 mmHg at baseline. A total of 924 (52.3%) participants were male and 173 (9.8%) participants had hypertension at baseline. During a mean of 7.2-year of follow up, the average SBP and DBP increased by 12.2 mm Hg and 8.4 mm Hg, respectively, and 512 (32.1%) participants free from hypertension at baseline developed hypertension.

Figure 1 and Supplementary Table 2 show the associations of 52 SNPs in Nox-related genes with BP changes and hypertension. SNPs rs12094228, rs16861188 and rs12066019 in *NCF2* were significantly associated with longitudinal changes in SBP ($P_{interaction} = 1.1 \times 10^{-3}$, 2.8×10^{-3} and 1.2×10^{-3}, respectively; Table 2). Each copy of the minor allele for marker rs12094228, rs16861188 and rs12066019 were associated with mean SBP increases of 0.26, 0.53 and 0.46 mmHg per year, respectively. SNPs

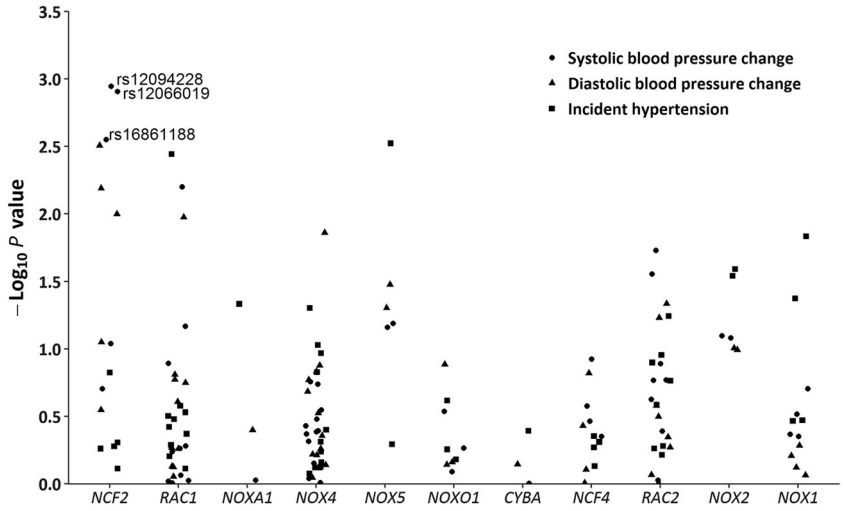

Fig. 1 Negative $\log_{10}$ *P*-values for the associations of 52 tag SNPs in NADPH oxidase-related genes with longitudinal changes in SBP and DBP, as well as incident hypertension (points were jittered to reduce overlap). The black circles and triangles show *P*-values for the testing of genotype by follow-up time interactions for SBP and DBP, respectively. The black squares show *P*-values for the testing of the effect of SNPs on hypertension. Three labeled SNPs were significantly associated with longitudinal changes in SBP after using false discovery rate procedures for multiple testing

rs12094228 and rs12066019 were nominally associated with longitudinal changes in DBP ($P_{\text{interaction}} = 6.5 \times 10^{-3}$ and 3.1×10^{-3}, respectively). Similar results were obtained after including kinships between individuals by the packages kinship2 and GWAF. For longitudinal BP change, sensitivity analyses excluding those participants with antihypertensive medicine revealed similar results (data not shown). There are no statistically significant associations of rs12094228, rs16861188, and rs12066019 with the risk of incident hypertension.

Table 3 presents the results of gene-based analyses. TPM and VEGAS have similar results and show that *NCF2* was significantly associated with SBP ($P_{\text{TPM}} = 1.00 \times 10^{-6}$, $P_{\text{VEGAS}} = 1.26 \times 10^{-4}$) and DBP changes ($P_{\text{TPM}} = 5.84 \times 10^{-4}$, $P_{\text{VEGAS}} = 1.04 \times 10^{-3}$). No genes was significantly associated with incident hypertension.

DISCUSSION

In this study, we investigated the association of the Nox-related genes with longitudinal BP changes and incident hypertension among a large sample of Han Chinese population. We identified three novel *NCF2* SNPs that were significantly associated with longitudinal SBP changes after correction for multiple testing. The minor alleles of rs12094228, rs16861188, and rs12066019 were related to an increased SBP change over time. Consistent with these findings, gene-based analyses also revealed an association of *NCF2* gene with BP changes over time.

The p67phox coded by *NCF2* is a core cytosolic component of Nox. Several studies have shown physiological evidence of the association between p67phox and BP control [26, 27]. For instance, in Dahl salt-sensitive (SS) rats, the higher expression of p67phox was associated with higher Nox activity and greater salt sensitivity; even with large increases of salt intake, suppression of p67phox produced significant reductions of hypertension, oxidative stress, and renal injury [26]. Further functional study showed that the kidney of SS rats was particularly vulnerable to oxidative stress, and a reduction of Nox2 in SS rats with a null mutation of p67phox resulted in a significant reduction of oxidative stress from mitochondria in the renal medulla [27]. In summary, these experiments demonstrated the role of p67phox in chronic BP regulation and salt sensitivity.

However, few studies evaluated the association between *NCF2* gene variants and longitudinal BP phenotypes. Our study provides the first evidence of an influence of *NCF2* gene on longitudinal BP phenotypes. The rs12066019 locates at 2 kb upstream of *NCF2*, whereas rs12094228 and rs16861188 are in the intronic region. We used RegulomeDB, HaploReg and SNPinfo to speculate the functional implication of the significant SNPs [28–30]. We found that rs12066019 locates at a potential transcription factor binding site for peroxisome proliferator-activated receptors (PPARs), which could regulate Nox activity and play an important role in the pathophysiology of hypertension [31]. A PhenoScanner [32] search of genotype-tissue expression (GTEx) project also showed that rs12066019 could affect the gene expression of *NCF2* in

Table 2 Association of SNPs with BP changes and incident hypertension among GenSalt study participants

gene	SNP	Chr	Position	Allele[a]	SBP		DBP		HTN	
					β	*P*(Q)	β	*P*(Q)	OR (95% CI)	*P*(Q)
NCF2	rs12094228	1	183547872	T/G	0.26	1.1×10^{-3} (0.03)[b]	0.15	6.5×10^{-3} (0.14)	1.06 (0.87, 1.30)	0.55 (0.72)
	rs16861188	1	183553326	T/C	0.53	2.8×10^{-3} (0.049)[b]	0.12	0.28 (0.61)	1.34 (0.90, 2.00)	0.15 (0.52)
	rs12066019	1	183561560	G/T	0.46	1.2×10^{-3} (0.03)[b]	0.27	3.1×10^{-3} (0.14)	1.06 (0.73, 1.54)	0.77 (0.79)

β coefficient, *Chr* chromosome, *CI* confidence interval, *DBP* diastolic blood pressure, *HTN* hypertension, *OR* odds ratio, *P* raw *P*-values, *Q* *Q*-values, *SBP* systolic blood pressure, *SNP* single-nucleotide polymorphism

[a] Major allele/minor allele

[b] False discovery rate *Q*-values that are less than 0.05

Table 3 Gene-based associations of NADPH oxidase-related genes with longitudinal BP phenotypes in the GenSalt study

Gene[a]	N	TPM			VEGAS		
		SBP	DBP	HTN	SBP	DBP	HTN
NCF2	5	1.00×10^{-6}[b]	5.84×10^{-4}[b]	0.27	1.26×10^{-4}[b]	1.04×10^{-3}[b]	0.56
RAC1	10	0.07	0.30	0.27	0.20	0.19	0.20
NOX4	12	0.60	0.36	0.29	0.60	0.22	0.37
NOX5	2	0.07	0.04	0.01	0.04	0.02	0.01
NOXO1	3	0.22	0.26	0.19	0.60	0.38	0.48
NCF4	4	0.19	0.38	0.17	0.25	0.52	0.77
RAC2	8	0.03	0.09	0.27	0.02	0.23	0.16
NOX2	2	0.08	0.10	0.03	0.09	0.10	0.03
NOX1	4	0.18	0.19	0.03	0.35	0.86	0.05

DBP diastolic blood pressure, *HTN* hypertension, *N* number of tag SNPs, *SBP* systolic blood pressure, *TPM* truncated product method, *VEGAS* versatile gene-based association study

[a] Genes containing at least two SNPs

[b] Significant after adjustment for multiple testing

skin, esophagus, mucosa, and colon transverse, etc. However, further functional studies are necessary to investigate the role of rs12066019 in BP progression. From these web tools, little evidence showed that rs12094228 and rs16861188 are causally associated with the regulation of *NCF2* expression. In addition to the single-marker analyses, *NCF2* was aggregately associated with longitudinal BP changes in gene-based analyses. With similar gene-based results from both TPM and VEGAS, our findings highlight the gene-based analyses for increasing statistical power to investigate potential genetic mechanisms underlying BP regulation. Further genetic or functional research are needed to investigate the mechanism of *NCF2* in BP regulation.

A recent GWAS meta-analysis showed that the minor A allele of SNP rs2289125 in the *NOX4* gene was associated with lower pulse pressure (PP) ($P = 9.1 \times 10^{-22}$) in European ancestry and correlated with increased *NOX4* expression in endothelial cells [5]; nevertheless, the association of rs2289125 with BP traits was not replicated in Asian [4]. Although genotype data were not available for rs2289125 in GenSalt study, two SNPs near rs2289125 with maximum linkage disequilibrium (rs595518 and rs3017887, $r^2 = 0.283$ and 0.28, respectively) were genotyped and not associated with SBP, DBP or PP in our analyses. Considering the different genetic background in East Asian and European and the inconsistent result from meta-analyses [4, 5], it is still uncertain whether rs2289125 is associated with BP traits in Chinese. Several studies showed that genetic variants in *CYBA* influenced the level of oxidative stress and were associated with hypertension [6, 14, 33]. For example, C242T (rs4673) in *CYBA* was associated with hypertension in Caucasian [33]. But only rs12709102 was available in the current study and in weak linkage disequilibrium with C242T ($r^2 = 0.02$), we could not confirm the effect of *CYBA* gene C242T on hypertension in Chinese.

Strengths and limitations

To our knowledge, this is the first study to examine associations of Nox-related genes with BP changes and the risk of incident hypertension in a cohort study. Second, considering the homogeneity of the population in GenSalt study, the result is robust to population stratification. Besides, except for slightly younger (35.5 vs. 39.0 years), individuals included in this analysis (92.8%) are similar in distribution of gender and genotypes, BMI and BP compared with those dropped out (Supplementary Table 3 and 4). In addition, we used rigorous quality control procedures to ensure high quality genotype and phenotype data, including measurements of BP and covariates. Moreover, false discovery rate procedures were performed to account for multiple testing. However, there are several limitations in this study. The novel findings in our study need to be replicated in an external population with different genetic background. Furthermore, even though the Affymetrix 6.0 platform has good genomic coverage of common variants in the Han Chinese population [34], in this study we have limited genotype data in *CYBA* and *NOXA1* genes. Thus, researches are still needed to examine these genes in future.

Finally, we do not provide in-vitro or in-vivo evidence for the biological function of these findings. Further studies are needed to investigate how the identified risk loci contribute to BP progression at the molecular and cellular level.

In conclusion, our study provided evidence that common variants of Nox-related genes were associated with longitudinal BP phenotypes in Han Chinese. The Noxs are major sources of ROS in human vessels. Since polymorphic variants in Nox-related genes may modulate ROS production and influence hypertension. The identification of polymorphisms in *NCF2* may be helpful to characterize patients with a genetically increased susceptibility to oxidative stress and hypertension, which is an important goal in human genetics research and the ear of precision medicine. However, replications of the findings in other populations are needed and functional studies are warranted to investigate the potential mechanism.

Summary

What is known about the topic?

- NADPH oxidases are major sources of reactive oxygen species, which play an important role in blood pressure regulation and the pathogenesis of hypertension.
- The associations between NADPH oxidase-related genes and blood pressure changes and incident hypertension have not been well examined.

What this study adds

- In Han Chinese population, *NCF2* SNPs rs12094228, rs16861188 and rs12066019 were significantly associated with longitudinal change in systolic blood pressure.
- Gene-based analyses revealed that *NCF2* gene was significantly associated with systolic blood pressure and diastolic blood pressure changes.

Acknowledgements This study is supported by the National Natural Science Foundation of China (81570386, 91439202, 91643208 and 81600332) and the CAMS Innovation Fund for Medical Sciences (grants no. 2017-I2M-1–004 and 2016-I2M-2–001). The GenSalt study is supported by a cooperative agreement project grant (U01HL072507, R01HL087263, and R01HL090682) from the National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, USA.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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