Genetic Variations in the Sodium Balance-Regulating Genes *ENaC*, *NEDD4L*, *NDFIP2* and *USP2* Influence Blood Pressure and Hypertension

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Key Words

ENaC · *NEDD4L* · *NDFIP2* · *USP2* · Blood pressure · Hypertension

Abstract

Background/Aims: In humans, the kidneys regulate blood pressure by balancing sodium concentrations. Fine-tuning of renal sodium reabsorption and excretion depends on the epithelial sodium channel protein (ENaC: protein complex of SCNN1A, SCNN1B, and SCNN1G). The surface expression of ENaC components is directed by the ubiquitination of ENaC by NEDD4L, an ENaC-specific E3 ubiquitin ligase, and is regulated by the deubiquitination of ENaC by USP2. The activity of NEDD4L in turn is regulated by phosphorylation by SGK1 and also through interaction with NDFIP2. **Methods:** We analyzed 91 SNPs in 7 genes using the genotype data of 8,842 individuals from the Korea Association REsource subject pool for their correlation with blood pressure and hypertension. **Results:** 25 SNPs in the *SCNN1A*, *SCNN1B*, *SCNN1G*, *NEDD4L*, *NDFIP2*, and *USP2* loci were found to be associated with blood pressure. An additional hypertension case-control study identified 13 SNPs in *SCNN1B*, *SCNN1G*, and *NEDD4L* that were linked to hypertension. **Conclusion:** These results support our hypothesis that individual variations in

blood pressure are attributed to variants of the genes that regulate renal sodium reabsorption and excretion. Our data also suggest that it would be meaningful to investigate the role of NEDD4L-mediated ubiquitination in the pathogenesis of hypertension.

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Introduction

Essential hypertension is caused by a complex interaction of environmental and genetic factors [1], but not all populations carry the same risk of developing hypertension [2]. Human blood pressure is controlled by the kidney, predominantly through the balance of salt concentrations. Fine-tuning of renal NaCl reabsorption and excretion involves the epithelial sodium channel (ENaC) that lines the distal nephron, and ENaC is critical for the homeostasis of sodium balance [3].

ENaC is a heteromultimeric protein complex that consists of three homologous subunits: α , β , and γ (SCNN1A, SCNN1B, and SCNN1G, respectively). Several Mendelian disorders that manifest as abnormal blood pressure are caused by sodium imbalances [4–7]. For example, Liddle syndrome is characterized by early and severe hy-

pertension and is caused by a mutation in the ENaC genes, which leads to a defect in ENaC ubiquitination and consequently the constitutive activation of ENaC [8].

The ubiquitination of ENaC is catalyzed by NEDD4L (neural precursor cell expressed, developmentally down-regulated 4-like), initiating ubiquitin-mediated endocytosis and lysosomal targeting of ENaC for its removal from the luminal cell membrane in renal collecting ducts [9, 10]. The *Nedd4-2* null mice were to produce salt-sensitive hypertension [11], and there was a correlation between *Nedd4-2* immunostaining and apical ENaC localization along the distal nephron of mice [12]. Therefore, polymorphisms in *NEDD4L* have been implicated in hypertension and blood pressure in many genome-wide scans [13–15], genetic studies [16–18], and a function study [19]. Presumably, genetic variations in *NEDD4L* influence renal sodium reabsorption and the resulting blood volume by modulating ENaC stability in the renal collecting ducts.

Serum/glucocorticoid-regulated kinase 1 (SGK1) is a serine/threonine protein kinase that regulates the binding of NEDD4L to ENaC; SGK1 binds and phosphorylates NEDD4L, reducing the latter's binding to ENaC [20, 21]. Consequently, SGK1 regulates epithelial Na⁺ transport in part by phosphorylation-dependent inhibition of NEDD4L. Expression of SGK1 results in the same phenotype as Liddle syndrome, wherein a mutation in the PPXY motif of ENaC disrupts its interaction with the WW domain of NEDD4L [14]. The hyperphosphorylation of NEDD4L by SGK1 increases ENaC expression on the cell membrane and the resulting predisposition to hypertension, whereas decreased phosphorylation has a protective effect [22].

Another molecule, *Nedd4* family-interacting protein 2 (NDFIP2), is highly expressed in collecting duct cells of the kidney that expresses ENaC, and it binds to NEDD4L via the WW domain [23]. NDFIP2 localizes not only on the Golgi apparatus but also on the endoplasmic reticulum and mitochondria [24]. Overexpression of NDFIP2 in *Xenopus laevis* oocytes upregulates the cell surface expression of ENaC [23]. Likely, NDFIP2-containing vesicles sequester NEDD4L, preventing its recruitment to ENaC at the plasma membrane. Little is known, however, about the association of genetic polymorphisms in the NDFIP2 locus and blood pressure control.

Recently the de-ubiquitination of ENaC by *Usp2-45*, an ortholog of ubiquitin-specific peptidase 2 (USP2) in man, was recognized in mice and cell systems [25, 26]. *Usp2-45* interferes with ENaC ubiquitination, and increases the total amount and function of ENaC [25].

In this study, using a population-based genome-wide association study, the Korea Association REsource (KARE) data, we examined genetic variations in sodium balance-controlling genes for their association with blood pressure. We identified SNPs in the loci of ENaC subunits that had a statistically significant association with blood pressure and in genes that regulate the ubiquitin-mediated turnover of ENaC. Our data confirm previous reports on the contribution of SNPs in *ENaC* and *NEDD4L*.

Notably, we also detected blood pressure-associated SNPs in *NDFIP2* and *USP2*, which can modulate the rate of ENaC ubiquitination. In light of the increasing attention that is being paid to the role of ubiquitin-mediated degradation in the fine-tuning of cellular processes, our data provide insight into the molecular pathogenesis of hypertension.

Materials and Methods

Subjects and Clinical Characteristics

Subjects in the KARE genome-wide association study were described by Cho et al. [27]. Briefly 10,038 persons from the Ansung-Ansan prospective community cohorts were recruited. The initial numbers of subjects aged 40–69 years from Ansung and Ansan were 5,018 and 5,020, respectively. Of the 10,038 subjects, 1,196 were excluded due to poor genotyping data. In addition, 1,291 subjects who were on hypertensive therapy or drug treatments that were likely to influence blood pressure were also excluded.

The remaining 7,551 subjects were investigated. Their basic characteristics, e.g., blood pressure and biochemical measures, are described in table 1. Blood pressure measurements were taken 3 times in the supine position, and the average value was used for the study. This study was approved by the institutional review board of the Korean National Institute of Health. Written informed consent was obtained from all subjects.

Genotyping and Selection of SNPs

The detailed genotyping and quality-control processes were reported in Cho et al. [27]. Briefly, most DNA samples were isolated from the peripheral blood of participants and genotyped using the Affymetrix Genome-Wide Human SNP array 5.0 (Affymetrix, Inc., Santa Clara, Calif., USA). The accuracy of the genotyping was calculated by Bayesian Robust Linear Modeling using the Mahalanobis distance (BRLMM) algorithm [28]. Samples that had genotyping accuracies of <98%, high missing genotype call rates (≥4%), high heterozygosity (>30%), or gender biases were excluded.

The SNPs that we analyzed were selected from the KARE data, based on their positions within the gene boundary (20 kb upstream and downstream of the first and last exons, respectively) (online suppl. table 1; for all online supplementary material, see www.karger.com/doi/10.1159/000275706). Ninety-one SNPs in 7 genes (*SCNN1A*, *SCNN1B*, *SCNN1G*, *NEDD4L*, *NDFIP2*, *SGK1*, and *USP2*) were selected. The positions of the SNPs were validated

in the NCBI database (<http://www.ncbi.nlm.nih.gov>). The clinical information and genotype data that we used were graciously provided by the Center for Genome Science, KNIH, KCDC.

Statistical Analysis

Most statistical analyses were performed using PLINK version 1.06 (<http://pngu.mgh.harvard.edu/~purcell/plink>) and PASW Statistics version 17.0 (SPSS Inc., Chicago, Ill., USA). Linear regression was used to analyze systolic blood pressure (SBP) and diastolic blood pressure (DBP) as quantitative traits in the final 7,551 subjects, controlling for cohort, age, sex, and body mass index (BMI) as covariates.

The 91 SNPs that we selected were also analyzed in hypertension case-control studies using logistic regression analysis. For case designations, the hypertensive cases ($n = 1,968$) were defined as SBP ≥ 140 mm Hg and/or DBP ≥ 90 mm Hg, including the 961 subjects who were treated with antihypertension drugs. Normotensive controls ($n = 4,452$) were defined as SBP < 120 mm Hg and DBP < 80 mm Hg, excluding subjects with prehypertensive status (120 mm Hg $<$ SBP < 140 mm Hg, and/or 80 mm Hg $<$ DBP < 90 mm Hg).

All association tests were based on an additive, dominant, or recessive model, and p values were not adjusted for multiple tests. Statistical significance was determined at a two-tailed value of $p < 0.05$. The Haploview version 4.1 (Whitehead Institute for Biomedical Research, Cambridge, Mass., USA) program was used to examine the structure of the linkage disequilibrium (LD) block [29] using the KARE genotype data for *NEDD4L* and the HapMap database (International HapMap Project, <http://www.hapmap.org/>) for other genes. We examined the LD coefficient r^2 between all pairs of biallelic loci [30].

Results

Quantitative Trait Analysis of Blood Pressure

The mean SBP and DBP in the 7,551 subjects was 115.65 ± 17.25 and 74.21 ± 11.27 mm Hg, respectively (table 1). Ninety-one SNPs in 7 genes (5 SNPs in *SCNN1A*, 9 in *SCNN1B*, 6 in *SCNN1G*, 60 in *NEDD4L*, 5 in *NDFIP2*, 3 in *SGKI*, and 3 in *USP2*) were selected and analyzed for their association with blood pressure.

Linear regression analysis was used to associate genotypes with SBP and DBP, controlling for age, sex, BMI, and cohort as covariates. The SNPs that had a statistically significant link are listed in table 2. Twenty-five SNPs in 6 genes had significant associations: 1 SNP in *SCNN1A* ($p = 0.047$ – 0.042), 2 in *SCNN1B* ($p = 0.044$ – 0.013), 2 in *SCNN1G* ($p = 0.040$ – 0.039), 16 in *NEDD4L* ($p = 0.049$ – 0.017), 3 in *NDFIP2* ($p = 0.040$ – 0.006), and 1 in *USP2* ($p = 0.040$ – 0.023). Subjects who harbored minor alleles of *SCNN1B*, *NEDD4L*, and *USP2* tended to have lower than the mean blood pressures, except for those who expressed rs17064520 of *NEDD4L*, and carriers of minor alleles of *SCNN1A*, *SCNN1G*, and *NDFIP2* tended to have blood pressures that were above the mean.

Table 1. Basic characteristics of the study subjects

Variables	
Total subjects	8,842
<i>Quantitative trait analysis</i>	
Number of individuals without hypertensive medication	7,551
Gender	
Men (%)	3,747 (49.6)
Women (%)	3,804 (50.4)
Age, years (mean $\pm$ SD)	51.44 ± 8.78
Body mass index (mean $\pm$ SD)	24.42 ± 3.07
Systolic blood pressure (SBP; mean $\pm$ SD)	115.65 ± 17.25
Diastolic blood pressure (DBP; mean $\pm$ SD)	74.21 ± 11.27
<i>Case-control analysis</i>	
Controls (SBP < 120 mm Hg and DBP < 80 mm Hg)	4,452
Cases (SBP ≥ 140 mm Hg and/or DBP ≥ 90 mm Hg and hypertensive medication)	1,968

Hypertension Case-Control Analysis

For the case-control analysis, we recruited 4,492 hypertension cases and 1,968 normotensive controls. Logistic regression analysis of the 91 SNPs in the 7 genes was performed to determine their link to hypertension status, controlling for age, sex, BMI, and cohort as covariates. The significantly associated SNPs are listed in table 3.

Thirteen SNPs in 3 genes (2 SNPs in *SCNN1B*, 3 in *SCNN1G*, and 8 in *NEDD4L*) were significantly associated in at least one genetic mode. Carriers of minor alleles of the statistically significant SNPs in *SCNN1B* and *NEDD4L* were resistant to the development of hypertension, except for those who had rs4941364 in *NEDD4L* [the lowest OR 0.54 (rs158869 in *NEDD4L*); the highest OR 0.91 (rs8044970 in *SCNN1B*)]. In contrast, carriers of minor alleles of *SCNN1G* were susceptible to hypertension (OR 1.63–1.72).

Nine SNPs in 3 genes (*SCNN1B*, *SCNN1G*, and *NEDD4L*, indicated in bold, table 3) showed a significant association with both blood pressure and hypertension. The minor alleles of 2 SNPs (rs7205273 and rs8044970) in *SCNN1B* and 5 SNPs (rs4941333, rs525839, rs292456, rs7243164, and rs12606138) in *NEDD4L* were associated with a decrease in SBP and DBP. As a consequence, carriers of these alleles had lower odds ratios in the case-control analysis. The minor alleles of 2 SNPs (rs4073930, and rs4073291) in *SCNN1G* contributed to higher mean SBPs and DBPs compared with other genotypes and also indicated a higher risk of hypertension.

Table 2. Linear regression analyses between 25 significant polymorphisms in six genes and blood pressure

Gene	SNP		Genotype			Additive P	Dominant P	Recessive P
			A1/A1	A1/A2	A2/A2			
SCNN1A	rs13306613	n	19	670	6,861			
		SBP	116.0 ± 14.28	115.2 ± 18.18	115.7 ± 17.16	0.254	0.283	0.507
		DBP	74.9 ± 10.69	73.5 ± 11.78	74.3 ± 11.22	0.047	0.042	0.868
SCNN1B	rs7205273	n	576	2,986	3,988			
		SBP	114.8 ± 17.04	115.6 ± 16.90	115.8 ± 17.53	0.044	0.084	0.121
		DBP	73.6 ± 11.35	74.3 ± 10.99	74.2 ± 11.47	0.190	0.444	0.092
	rs8044970	n	1,468	3,632	2,439			
		SBP	115.2 ± 17.16	115.5 ± 17.03	116.1 ± 17.63	0.019	0.013	0.201
		DBP	73.8 ± 11.07	74.3 ± 11.25	74.4 ± 11.42	0.084	0.242	0.088
SCNN1G	rs4073930	n	153	1,697	5,686			
		SBP	117.4 ± 18.21	115.7 ± 17.40	115.6 ± 17.18	0.428	0.640	0.194
		DBP	75.9 ± 12.13	74.0 ± 11.48	74.2 ± 11.17	0.771	0.728	0.039
	rs4073291	n	153	1,765	5,632			
		SBP	117.4 ± 18.21	115.7 ± 17.35	115.6 ± 17.19	0.457	0.676	0.195
		DBP	75.9 ± 12.13	74.0 ± 11.41	74.2 ± 11.20	0.717	0.797	0.040
NEDD4L	rs483540	n	13	653	6,884			
		SBP	103.2 ± 12.63	116.3 ± 17.21	115.6 ± 17.25	0.749	0.537	0.049
		DBP	68.8 ± 11.71	74.4 ± 11.49	74.2 ± 11.25	0.996	0.880	0.284
	rs4941333	n	193	2,006	5,348			
		SBP	113.1 ± 16.54	115.6 ± 16.93	115.8 ± 17.39	0.244	0.529	0.044
		DBP	73.1 ± 10.90	74.3 ± 11.24	74.2 ± 11.30	0.701	0.927	0.128
	rs12960965	n	419	2,621	4,506			
		SBP	114.1 ± 16.26	115.6 ± 17.13	115.8 ± 17.39	0.120	0.340	0.043
		DBP	73.7 ± 10.98	74.2 ± 11.19	74.3 ± 11.33	0.441	0.574	0.418
	rs525839	n	1,624	3,683	2,241			
		SBP	115.3 ± 16.23	115.9 ± 17.45	115.5 ± 17.61	0.856	0.300	0.401
		DBP	74.2 ± 10.65	74.4 ± 11.45	73.8 ± 11.40	0.115	0.030	0.755
	rs292456	n	317	2,487	4,737			
		SBP	113.8 ± 15.21	116.0 ± 17.47	115.6 ± 17.26	0.408	0.900	0.040
		DBP	73.3 ± 9.97	74.5 ± 11.26	74.1 ± 11.36	0.920	0.666	0.185
	rs17064520	n	1,623	3,692	2,235			
		SBP	116.5 ± 16.56	115.7 ± 17.25	115.0 ± 17.71	0.017	0.068	0.036
		DBP	74.6 ± 10.93	74.2 ± 11.26	73.9 ± 11.52	0.120	0.264	0.147
	rs8093609	n	39	1,028	6,483			
		SBP	113.2 ± 15.31	115.5 ± 17.09	115.7 ± 17.28	0.420	0.436	0.724
		DBP	71.3 ± 11.19	73.8 ± 11.11	74.3 ± 11.29	0.047	0.065	0.231
	rs9955907	n	42	1,069	6,439			
		SBP	114.1 ± 14.63	115.3 ± 17.14	115.7 ± 17.28	0.303	0.269	0.938
		DBP	72.5 ± 10.82	73.7 ± 11.24	74.3 ± 11.28	0.036	0.034	0.622
	rs8093462	n	66	1,224	6,260			
		SBP	114.1 ± 13.49	115.1 ± 17.10	115.8 ± 17.31	0.205	0.185	0.865
		DBP	73.0 ± 9.97	73.5 ± 11.18	74.4 ± 11.30	0.025	0.020	0.687
	rs7228980	n	61	1,297	5,912			
		SBP	113.4 ± 13.58	115.1 ± 17.08	115.8 ± 17.31	0.221	0.206	0.847
		DBP	72.7 ± 10.16	73.6 ± 11.19	74.4 ± 11.28	0.022	0.019	0.649
	rs7243164	n	99	1,493	5,953			
		SBP	112.5 ± 13.32	115.1 ± 17.15	115.8 ± 17.32	0.059	0.091	0.173
		DBP	72.6 ± 9.76	73.6 ± 11.42	74.4 ± 11.25	0.019	0.021	0.333
	rs12606138	n	73	1,284	6,193			
		SBP	113.6 ± 14.76	115.3 ± 17.29	115.8 ± 17.26	0.314	0.311	0.765
		DBP	72.6 ± 10.22	73.6 ± 11.17	74.4 ± 11.30	0.035	0.033	0.532

Table 2 (continued)

Gene	SNP		Genotype			Additive P	Dominant P	Recessive P
			A1/A1	A1/A2	A2/A2			
	rs9962106	n	695	3,169	3,685			
		SBP	114.9 ± 17.50	115.3 ± 17.02	116.1 ± 17.38	0.077	0.044	0.613
		DBP	73.8 ± 11.49	74.1 ± 11.33	74.4 ± 11.18	0.279	0.246	0.667
	rs12457509	n	540	2,972	4,037			
		SBP	115.0 ± 17.49	115.2 ± 16.96	116.1 ± 17.41	0.035	0.021	0.504
		DBP	74.1 ± 11.34	73.9 ± 11.31	74.4 ± 11.22	0.208	0.114	0.997
	rs10513914	n	539	2,944	4,067			
		SBP	114.8 ± 17.42	115.2 ± 16.95	116.1 ± 17.42	0.031	0.028	0.329
		DBP	74.1 ± 11.23	74.0 ± 11.33	74.4 ± 11.23	0.269	0.177	0.942
	rs3744865	n	542	2,883	4,096			
		SBP	114.7 ± 17.53	115.3 ± 16.98	116.1 ± 17.41	0.034	0.029	0.355
		DBP	74.0 ± 11.32	74.0 ± 11.32	74.4 ± 11.24	0.301	0.205	0.946
NDFIP2	rs12858106	n	388	2,610	4,545			
		SBP	117.2 ± 15.20	116.1 ± 17.54	115.3 ± 17.23	0.009	0.011	0.169
		DBP	75.8 ± 10.47	74.4 ± 11.32	74.0 ± 11.29	0.006	0.024	0.015
	rs2783122	n	386	2,623	4,534			
		SBP	116.9 ± 15.18	116.1 ± 17.51	115.3 ± 17.26	0.024	0.028	0.237
		DBP	75.6 ± 10.44	74.4 ± 11.34	74.0 ± 11.29	0.010	0.034	0.026
	rs1408049	n	390	2,554	4,478			
		SBP	116.9 ± 15.15	116.0 ± 17.5	115.4 ± 17.28	0.040	0.051	0.240
		DBP	75.6 ± 10.40	74.4 ± 11.36	74.0 ± 11.30	0.015	0.051	0.027
USP2	rs2241646	n	182	2,007	5,359			
		SBP	113.5 ± 17.67	115.5 ± 17.10	115.8 ± 17.29	0.040	0.120	0.023
		DBP	73.3 ± 11.16	74.0 ± 11.40	74.3 ± 11.23	0.080	0.141	0.130

Blood pressure was measured in the supine position, controlling for age, sex, BMI and cohort as covariates. Statistical significance ($p < 0.05$) is indicated by bold type.

Linkage Disequilibrium Analysis of NEDD4L

Nineteen statistically significant SNPs in *NEDD4L* were analyzed for LD to address the involvement of multiple versus single genetic variants. The 60 SNPs of *NEDD4L* were divided into 11 LD blocks that were generated from the KARE genotype data (fig. 1). Most of the statistically significant SNPs were spread throughout the blocks, suggesting that individual variations in blood pressure are attributed to more than one type of genetic variation in *NEDD4L*.

Notably, several significant SNPs gathered in one particular LD block (10th block) which contains the WW domains (exon 13–20) that are important for the interaction between ENaC, SGK1, and NDFIP2. The 10th block also includes a site (Ser444) in *NEDD4L* that can be phosphorylated by SGK1, as well as the HECT domain, which corresponds to the catalytic site of the E3 ubiquitin ligase.

The LD blocks and the locations of significant SNPs in other genes are depicted over their gene structures (online suppl. fig. 1).

Age-Dependent Association of NEDD4L SNPs with Blood Pressure and Hypertension

Two of the SNPs in *NEDD4L* (rs182383 and rs3865418) were reanalyzed in the age subgroups of the original subjects; there were 3,863, 1,901, and 1,787 subjects in their 40s, 50s, and 60s, respectively, because these SNPs are positively associated with blood pressure and hypertension in other populations. The rs182383 SNP was linked to hypertension in the subgroup aged in their 50s (dominant $p = 0.017$), and rs3865418 was associated with hypertension in the subgroup aged in their 40s (dominant $p = 0.026$; table 4).

Table 3. Logistic regression analysis of hypertension cases and controls using SNPs in *ENaC* and related genes (controlling for age, sex, BMI, and cohort as covariates)

Gene	SNP	Additive		Dominant		Recessive	
		OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p
<i>SCNN1B</i>	rs7205273	0.87 (0.79–0.96)	0.005	0.88 (0.78–0.99)	0.029	0.74 (0.58–0.93)	0.011
	rs8044970	0.91 (0.83–0.99)	0.027	0.89 (0.78–1.01)	0.065	0.87 (0.75–1.01)	0.073
<i>SCNN1G</i>	rs4480807	1.03 (0.91–1.16)	0.646	0.98 (0.86–1.12)	0.772	1.63 (1.10–2.41)	0.015
	rs4073930	0.99 (0.88–1.12)	0.916	0.93 (0.81–1.07)	0.332	1.69 (1.14–2.52)	0.010
	rs4073291	1.00 (0.89–1.13)	0.997	0.94 (0.82–1.08)	0.379	1.72 (1.16–2.56)	0.007
<i>NEDD4L</i>	rs4941333	0.99 (0.88–1.11)	0.829	1.03 (0.91–1.18)	0.640	0.67 (0.45–0.99)	0.045
	rs12965133	0.99 (0.88–1.11)	0.834	1.03 (0.90–1.18)	0.645	0.67 (0.45–1.00)	0.048
	rs4941364	1.08 (0.99–1.18)	0.081	1.14 (1.01–1.29)	0.042	1.05 (0.90–1.24)	0.515
	rs525839	0.95 (0.87–1.03)	0.231	1.03 (0.90–1.17)	0.703	0.83 (0.71–0.96)	0.012
	rs292456	0.93 (0.83–1.03)	0.150	0.95 (0.84–1.08)	0.454	0.70 (0.52–0.95)	0.024
	rs158869	0.98 (0.86–1.11)	0.726	1.02 (0.89–1.17)	0.751	0.54 (0.33–0.90)	0.019
	rs7243164	0.85 (0.74–0.98)	0.021	0.84 (0.72–0.98)	0.024	0.74 (0.42–1.31)	0.303
	rs12606138	0.86 (0.74–0.99)	0.040	0.85 (0.72–0.99)	0.040	0.80 (0.44–1.48)	0.480

Cases (n = 4,452) were subjects with SBP <120 mm Hg and DBP <80 mm Hg, and controls (n = 1,968) were subjects with SBP ≥140 mm Hg and/or DBP ≥90 mm Hg, and hypertension therapy. Odds ratios that are statistically significant (p < 0.05) are indicated by bold type.

Discussion

SNPs in genes that control renal reabsorption of sodium (ENaC subunits *SCNN1A*, *SCNN1B*, and *SCNN1G*) and their regulators (*NEDD4L*, *NDFIP2*, *SGK1*, and *USP2*) were examined for their association with blood pressure and hypertension using 7,551 subjects in the KARE study. The genes that we studied had been examined for their link to variations in blood pressure (online suppl. table 3 and fig. 1).

None of the SNPs in the ENaC subunit genes (*SCNN1A*, *SCNN1B*, and *SCNN1G*) overlapped with the SNPs whose association with blood pressure has been reported. Because other studies were performed with non-Korean populations, this disparity may reflect differences in SNP profiles between ethnic groups. Nevertheless, the rich list of SNPs that are associated with hypertension and blood pressure in Europeans [31, 32], Japanese [33], and Americans [34, 35] underscores the prominent role of ENaC in the maintenance of normal blood pressure.

With regard to the *NEDD4L* gene, 3 of the SNPs that we analyzed have been reported to be associated with blood pressure in other populations. Russo et al. [36] reported that rs7228980 and rs182383 are linked to blood pressure in African-Americans, and that rs3865418 is associated with hypertension in Caucasians in the US and

Greece (online suppl. table 3). A correlation between rs3865418 and hypertension has also been reported in Han Chinese [37]. We did not, however, observe any significant associations between blood pressure or hypertension and rs182383 and rs3865418; but rs7228980 was linked to blood pressure (table 2).

To explain this discrepancy, an association analysis was performed for subgroups that were divided by ages. Subjects in the study by Russo et al. [36] were aged 55 years or older, and the mean age of subjects who were examined by Wen et al. [37] was 56.2 ± 11.3 years; the mean age of subjects in this study was 51.44 ± 8.78 years – rs182383 was associated with hypertension in the subgroup aged in their 50s (dominant p = 0.017), and rs3865418 was linked to hypertension in the subgroup aged in their 40s (dominant p = 0.026; table 4). These results suggest that the association of SNPs with disease is influenced by the age of the subject, presumably due to physiological changes in the body.

Prior to this study, genetic variations in the *NDFIP2* and *USP2* genes had not been reported for any disease or dysfunction. In this study, we found that 3 SNPs in *NDFIP2* (rs12858106 in upstream SNP; rs2783122 in intron 2; rs1408049 in intron 3) and 1 synonymous SNP in *USP2* (rs2241646 in exon 5) were associated with blood pressure. The carboxyl terminus of each ENaC subunit

Fig. 1. Linkage disequilibrium and mapping analysis of NEDD4L. The top panel shows the physical position of NEDD4L on chromosome 18. The middle panel shows the positions and rs numbers of the 60 tested SNPs. The bottom panel shows a Haploview of LD (r^2) based on genotyping data from 7,551 KARE subjects. In the middle panel, 21 statistically significant SNPs of KARE subjects and from previous reports are in bold and located in 8 LD blocks, as follows: 1 = [rs483540-S]; 2 = [rs4941333-S,H, rs12965133-H];

3 = [rs12960965-S]; 5 = [rs4941364-H, rs525839-D,H, rs292456-S,H,rs182383-H*]; 7=[rs158869-H]; 7 and 8 interval=[rs17064520-S]; 8 = [rs8093609-D, rs9955907-D, rs8093462-D]; 9 = [rs7228980-D,H*, rs7243164-D,H, rs12606138-D,H] = 10 = [rs3865418-D*,H*, rs9962106-S, rs12457509-S, rs10513914-S, rs3744865-S]. S = Systolic blood pressure; D = diastolic blood pressure; H = hypertension. * SNPs previously reported to be associated.

Table 4. Logistic regression analysis of rs182383 and rs3865418 of NEDD4L and hypertension, controlling for age, sex, BMI and cohort as covariates

SNP	Age	Number of controls and cases	Additive		Dominant		Recessive	
			OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p
rs182383	Total	4,492/1,968	0.92 (0.84–1.01)	0.087	0.89 (0.79–1.01)	0.069	0.93 (0.74–1.15)	0.493
	40s	2,732/488	0.98 (0.83–1.15)	0.810	1.00 (0.81–1.22)	0.986	0.90 (0.60–1.34)	0.593
	50s	1,005/611	0.83 (0.71–0.99)	0.034	0.77 (0.62–0.95)	0.017	0.88 (0.60–1.29)	0.524
	60s	715/869	0.93 (0.79–1.09)	0.365	0.89 (0.72–1.10)	0.290	0.96 (0.67–1.39)	0.841
rs3865418	Total	4,492/1,968	1.01 (0.93–1.11)	0.765	1.01 (0.90–1.14)	0.814	1.02 (0.85–1.24)	0.789
	40s	2,732/488	1.11 (0.96–1.30)	0.141	1.26 (1.03–1.55)	0.026	0.93 (0.67–1.30)	0.687
	50s	1,005/611	0.92 (0.79–1.08)	0.332	0.88 (0.71–1.10)	0.261	0.95 (0.68–1.31)	0.744
	60s	715/869	0.99 (0.84–1.16)	0.866	0.91 (0.73–1.12)	0.358	1.24 (0.87–1.76)	0.243

Statistical significance ($p < 0.05$) is indicated by bold type.

contains a PPXY motif that is important for interaction with the WW domains of NEDD4L [23]. Disruption of this interaction, which occurs in Liddle syndrome, increases ENaC activity and arterial hypertension.

NDFIP2 regulates the surface expression of ENaC by binding to the WW domain of NEDD4L through its PY motif. Among the 3 associated SNPs of *NDFIP2*, rs12858106 had the highest level of significance (table 2). We found a potential NF- κ B-binding site near rs12858106, which is located in the 5' flanking region (8,342 bp upstream of the first exon; data not shown). The G-to-T nucleotide substitution, however, is unlikely to affect the binding of the transcription factor, according to the TRANSFAC database (<http://www.cbrc.jp/research/db/TFSEARCH.html>). It is unclear how causative variations influence blood pressure levels. Nevertheless, our data represent another example that a polymorphism of a gene that regulates the ubiquitination of ENaC is associated with blood pressure.

Usp2-45, an ortholog of USP2 in human, was recently identified as ENaC de-ubiquitination enzyme, and it was suggested that Usp2-45 interfered with the NEDD4L-dependent ubiquitination regulation. In human, USP2 function is not clear yet, though the homology between human and mouse in USP2 is high (97% in density). This study provides evidence that a genetic variation in USP2 gene was associated with the SBP, suggesting the involvement of USP2 in de-ubiquitination of ENaC in human as well.

Previously, Busjahn et al. [38] identified 2 polymorphisms in the *SGK1* gene that correlated with elevated blood pressure in a twin study of German Caucasians: one synonymous polymorphism in exon 8 (rs1057293) and one in intron 6 (rs1743966). Trochen et al. [39] investigated the same 2 polymorphisms, however, and concluded that they do not exert any significant effect on blood pressure in Caucasians. Our study showed that

both rs1057293 and rs1743966 are associated with blood pressure in women in their 50s ($n = 941$; rs1057293: additive $p = 0.034$, dominant $p = 0.0036$; and rs1743966: dominant $p = 0.036$; data not shown).

In other contemporary studies, the subjects in the study by Busjahn et al. [38] were monozygotic twins ($n = 252$, age 29 ± 12 years) and dizygotic twins ($n = 140$, age 31 ± 12 years) and in the study by Trochen et al. [39] they were healthy normotensive individuals ($n = 167$, age 49 ± 16 years) and hypertensive individuals ($n = 113$, age 59 ± 17 years). It is possible that polymorphisms in *SGK1* influence blood pressure only in certain age groups.

In summary, we investigated the presence of blood pressure- and hypertension-associated SNPs in genes that encode the ENaC α , β , and γ subunits and proteins that regulate their ubiquitination and de-ubiquitination. In *SCNN1A*, *SCNN1B*, *SCNN1G*, *NEDD4L*, *NDFIP2*, and *USP2*, we found statistically significant SNPs that are associated with blood pressure. SNPs in *SCNN1B*, *SCNN1G*, and *NEDD4L* are also associated with hypertension. Our results further confirm that the controlled, ubiquitin-mediated degradation of renal reabsorption proteins is important for the maintenance of blood pressure and that individual differences in blood pressure and susceptibility to hypertension are attributed to the variants of genes that regulate this process.

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