Rare Exome Sequence Variants in \textit{CLCN6} Reduce Blood Pressure Levels and Hypertension Risk

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**Background**—Rare genetic variants influence blood pressure (BP).

**Methods and Results**—Whole-exome sequencing was performed on DNA samples from 17956 individuals of European ancestry and African ancestry (14497, first-stage discovery and 3459, second-stage discovery) to examine the effect of rare variants on hypertension and 4 BP traits: systolic BP, diastolic BP, pulse pressure, and mean arterial pressure. Tests of \( \approx 170 000 \) common variants (minor allele frequency, \( \geq 1\% \); statistical significance, \( P \leq 2.9\times 10^{-7} \)) and gene-based tests of rare variants (minor allele frequency, \( <1\% \); \( \approx 17 000 \) genes; statistical significance, \( P \leq 1.5\times 10^{-6} \)) were evaluated for each trait and ancestry, followed by multiethnic meta-analyses. In the first-stage discovery, rare coding variants (splicing, stop-gain, stop-loss, nonsynonymous variants, or indels) in \textit{CLCN6} were associated with lower diastolic BP (cumulative minor allele frequency, 1.3\%; \( \beta = -3.20 \); \( P = 4.1\times 10^{-6} \)) and were independent of a nearby common variant (rs17367504) previously associated with BP. \textit{CLCN6} rare variants were also associated with lower systolic BP (\( \beta = -4.11 \); \( P = 2.8\times 10^{-4} \)), mean arterial pressure (\( \beta = -3.50 \); \( P = 8.9\times 10^{-6} \)), and reduced hypertension risk (odds ratio, 0.72; \( P = 0.017 \)). Meta-analysis of the 2-stage discovery samples showed that \textit{CLCN6} was associated with lower diastolic BP at exome-wide significance (cumulative minor allele frequency, 1.1\%; \( \beta = -3.30 \); \( P = 5.0\times 10^{-7} \)).

**Conclusions**—These findings implicate the effect of rare coding variants in \textit{CLCN6} in BP variation and offer new insights into BP regulation. (\textit{Circ Cardiovasc Genet}. 2016;9:64-70. DOI: 10.1161/CIRCGENETICS.115.001215.)

**Key Words:** blood pressure ■ exome ■ genetic variation ■ genome-wide association study ■ hypertension

Blood pressure (BP) is a heritable quantitative trait influenced by both genetic and environmental stimuli.\(^1,2\) Persistently elevated BP is a risk factor of cardiovascular disease and a major contributor to cardiovascular death.\(^3,4\) Identifying genetic determinants of BP regulation may add novel insights into cardiovascular disease prevention and may lead to more efficacious treatments. Large-scale genome-wide association studies (GWAS) have reported common variants at \( \approx 60 \) loci that are associated with systolic BP (SBP) and diastolic BP (DBP) in individuals of European ancestry (EA), with effect sizes ranging from 0.4 to 1.2 mm Hg for SBP and 0.2 to 0.7 mm Hg for DBP per copy of the minor allele.\(^5,7\) Additional variants for pulse pressure (PP) and mean arterial pressure (MAP) have also been identified with effect sizes of similar magnitudes.\(^8\) A recent large BP GWAS demonstrated that BP variants identified in EAs may have effects in individuals of African ancestry; so, an analysis of multiethnic samples has the potential to find novel genetic determinants of BP traits in this field.\(^9\) Despite the fact that numerous BP variants have been identified by GWAS, the proportion of explained variance in BP measures remains limited.

**Clinical Perspective on p 70**

Studies have shown that rare coding mutations contribute to BP variation,\(^10,11\) but a recent study involving targeted sequencing of 6 BP genes identified by GWAS did not reveal novel rare
variants associated with the trait. In contrast, whole-exome sequencing (WES), which captures both common and rare coding variations, has successfully been applied to identify rare coding variants contributing to multiple complex traits. To date, no WES study has evaluated the association between rare coding variants and BP traits. To address this, we performed first-stage WES on 9950 EAs and 4547 black individuals from 6 large population-based cohort studies to examine the effect of rare coding variants on SBP, DBP, PP, MAP, and hypertension. The second-stage WES was conducted in 2 EA cohorts, comprising 3459 individuals.

Methods

Study Populations and BP Measurements

The first-stage discovery sample consisted of 10403 individuals from the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium17 and 4094 individuals from the National Heart, Lung, and Blood Institute Go Exome Sequencing Project (ESP) with BP measures. Individuals from CHARGE were from 3 population-based cohorts, including the Atherosclerosis Risk in Communities (ARIC) study (n=5704 EAs and 2792 blacks), the Cardiovascular Health Study (CHS; n=680 EAs), and the Framingham Heart Study (FHS; n=1227 EAs). Independent individuals from ESP were sampled from 6 population-based cohorts: ARIC (n=512 EAs and 323 blacks), CHS (n=144 EAs and 64 blacks), FHS (n=404 EAs), Jackson Heart Study (JHS; n=359 blacks), Multi-Ethnic Study of Atherosclerosis (MESA; n=247 EAs and 151 blacks), and the Women’s Health Initiative (WHI; n=1032 EAs and 858 blacks). The detailed sampling strategy for ESP is described in Methods section in the Data Supplement. The second-stage discovery sample consisted of individuals from the Rotterdam Study (RS; n=2205 EAs) and the Erasmus Rucphen Family (ERF) study (n=1254 EAs). Detailed descriptions of each of the 8 cohorts have been published elsewhere.17-24

For all cohorts in this study, BP values were measured at the first examination and antihypertensive medication use was recorded from the medication history or medication inventory at the same time. Detailed descriptions for BP measurements in each cohort are summarized in Methods section in the Data Supplement. For individuals taking antihypertensive medication, untreated BP values were imputed by adding 15 mm Hg to measured SBP and 10 mm Hg to measured DBP.25 All participants provided written informed consent as approved by local institutional review committees.

Exome Sequencing and Variant Calling

For CHARGE, DNA samples were prepared using the Baylor College of Medicine Human Genome Sequencing Center VCRome 2.1 design26 (42Mb; NimbleGen) and were sequenced and called together. For ESP, DNA samples were prepared using either Roche Nimblen SequaCap EZ or Agilent SureSelect Human All Exon 50Mb. All samples were paired end sequenced using Illumina GAII or HiSeq instruments. Details on sequencing, variant calling, and variant quality control are provided in Methods section in the Data Supplement.

Annotation of Whole-Exome Sequence Variants

To facilitate meta-analysis between CHARGE and ESP, a combined variant annotation file was created to include all quality variants observed in either CHARGE or ESP. Variants were annotated from CHARGE and ESP separately using ANNOVAR27 and dbNSFP version 2.028 according to the reference genome GRCh37 and National Center for Biotechnology Information RefSeq. Coding variants were annotated to a unique gene, as well as the following categories that were considered for inclusion in gene-based tests: splicing, stop-gain, stop-loss, non-synonymous variants, and indels. The CHARGE and ESP annotated variant lists were merged into a joint file to ensure that a variant present in both studies had the same reference allele and annotation category.

Statistical Analyses

Individuals with untreated SBP <60 mm Hg or untreated DBP <40 mm Hg were excluded from analysis. PP was calculated by subtracting DBP from SBP, and MAP was defined as DBP plus PP/3. Hypertension was defined as individuals having SBP ≥140 mmHg or DBP ≥90 mmHg or use of BP-lowering medication at the first examination. All 4 continuous traits were winzorized at the 99.9th percentile before the analysis by using BP data available from the entire cohort. Cohort-level and ancestry-specific analyses were carried out using the seqMeta package (http://cran.r-project.org/web/packages/seqMeta/index.html) adjusting for age, age-squared, sex, body mass index, and principal components (generated by EIGENSTRAT) or study site as needed within each cohort and ancestry stratum. Fixed-effect inverse variance–weighted meta-analyses of single-variant and gene-based tests were then conducted using seqMeta to combine cohort-level and ancestry-specific summary results for multietnic analyses. Only variants on autosomal chromosomes were analyzed in this study, and all analyses used additive genetic models.

Single variants (common variants; minor allele frequency [MAF], ≥1%) were tested for association with the 4 BP traits and hypertension. Single-variant associations were considered to be significant if P≤2.9×10–6, reflecting Bonferroni correction for testing 170 000 variants. For gene-based analysis, we performed a T1 test for each gene, in which annotated coding variants with MAF ≤1% within a gene were collapsed into a single-gene-based burden score, and then, the score was analyzed using linear regression.29 We also implemented the Sequence Kernel Association Test using default β weights,30 which analyzed annotated coding variants with an MAF of ≤1% and is more powerful when effects are both BP raising and BP lowering. For multiethnic meta-analyses, genes with cumulative MAF (cMAF) ≥0.1% were analyzed using both T1 test and Sequence Kernel Association Test implemented by seqMeta, and an association was considered to be significant if P≤1.5×10–5 given a Bonferroni correction for n=17 000 genes and 2 burden tests.

Second-Stage Discovery

The top T1 gene-based association identified in this study was followed up in 2 independent sample sets, RS (n=2205 EAs) and ERF (n=1254 EAs). For ERF, sequencing was done using the Agilent version V4 capture kit on an Illumina Hiseq2000 sequencer. In the RS, individuals were sequenced using the Nimblen SequaCap EZ V2 capture kit on an Illumina Hiseq2000 sequencer. Details on sequencing, variant calling, and variant quality control are provided in Methods section in the Data Supplement. Coding variants included in the analyses were defined as splicing, stop-gain, stop-loss, non-synonymous, and indels. A gene-based T1 test was conducted as described above, with the significance threshold set at P<0.05.

Results

Participant Characteristics

The study sample for this analysis consisted of 17 956 individuals, with 14 497 in the first-stage discovery data set and 3459 in the second-stage discovery data set. In general, individuals from each cohort were middle aged, with a greater proportion of women than men. Compared with EAs, blacks had higher prevalence of hypertension, type 2 diabetes mellitus, and higher mean body mass index and BP values. Ancestry-stratified characteristics of the 2-stage discovery cohorts are summarized in Table I in the Data Supplement.

Gene-Based Test Results

For each BP trait, the first-stage discovery results from T1 and Sequence Kernel Association Test gene-based tests at P<5×10–4 and rare coding variants in the identified genes are summarized in Tables II and III in the Data Supplement. The
most significant association was for the chloride channel, voltage-sensitive 6 gene (CLCN6) with DBP, in the T1 test. There were 95 rare coding variants in CLCN6 present in CHARGE or ESP (cMAF, 1.3%; annotated variant level results with DBP are shown in Table IV in the Data Supplement); 34 of which were not reported by the Exome Aggregation Consortium (http://exac.broadinstitute.org, Accessed March 31, 2015). The aggregation of rare coding variants in CLCN6 was associated with lower DBP (β=−3.20; P=4.1×10−6), SBP (β=−4.11; P=2.8×10−4), and MAP (β=−3.50, P=8.9×10−4) but was not associated with PP. Rare coding variants were seen in both ancestries with similar cMAF of 1.2% (Figure 1). The magnitude of the effect sizes was consistent between EA and blacks, where each copy of a rare allele was associated with 3 to 4 mm Hg lower DBP (Table 1). There were 29 BP genetic loci, including 42 genes, previously reported by Ehret et al, the largest BP GWAS thus far. Tables V and VI in the Data Supplement contain T1 and Sequence Kernel Association Test results for the 42 genes and the 4 BP traits. After accounting for multiple testing for the 42 genes (P<0.001), only CLCN6 exceeded this significance threshold.

In a T1 burden test for hypertension in the first-stage discovery sample, rare coding variants in CLCN6 accounted for a 28% lower odds of hypertension (odds ratio, 0.72; 95% confidence interval, 0.55–0.94; P=0.017). CLCN6 is located in 1p36. A common intronic single-nucleotide polymorphism (SNP), rs17367504 (MAF, 14%), 3.4 kb upstream from CLCN6, was associated with reduced DBP in a previous GWAS. Therefore, we re-examined the association between CLCN6 and DBP in CHARGE EAs and blacks, adjusting for rs17367504. The results showed the observed effect size, and significance of CLCN6 on DBP levels had the same magnitude as in the unconditional analyses (Table 2).

Corroborating Evidence

There are 3 sources of corroborating data for the observed first-stage discovery findings: second-stage discovery, previous GWAS, and animal model studies. When compared with the first-stage discovery cohorts, the 2-EA second-stage cohorts had a smaller cMAF (cMAF, 0.3% versus 1.3%) for CLCN6 in the T1 test, but the direction of the effect was consistent. CLCN6 remained significantly associated with DBP in ERF (β=−7.25; P=0.04) but not in RS (β=−1.19; P=0.68; variant level results are shown in Table VII in the Data Supplement; T1 results for the other BP traits are shown in Table VIII in the Data Supplement). After meta-analyzing the 2-stage discovery samples, CLCN6 was exome wide significantly related to lower DBP (cMAF, 1.1%; β=−3.30; P=5.0×10−5; Figure 2). Second, CLCN6 is near a previous DBP GWAS locus that contains multiple candidate genes. Third, a knock-out homologue Clcn6 in the rat results in reduced BP levels and lower hypertension risk, supporting results similar to our observations.

Single-Variant Test Results

Common variants (MAF≥1%) were analyzed in relation to BP traits using multiethnic meta-analyses. No single-variant test reached our predefined significance threshold. The associations for each BP trait with P<5×10−8 are shown in Table IX in the Data Supplement. Four coding variants located in ULK4, SLC39A8, HFE, and SH2B3 previously reported by Ehret et al, the largest BP GWAS thus far, were captured in this study, and thus, were available for analysis. Our results showed consistent directional effects for the coded alleles with the GWAS findings, and the associations with DBP all had P<0.05 (Table X in the Data Supplement).

Discussion

By analyzing exome sequence data from 2 large consortia (n=14497) in relation to BP traits, we identified an aggregation of rare coding variants in CLCN6 that were associated with lower DBP among EAs and blacks. The association was corroborated in the second-stage discovery cohorts, and a meta-analysis of 2-stage discovery cohorts showed that CLCN6 was exome wide significantly related to lower DBP (P=5.0×10−5). In addition to DBP, CLCN6 was related to lower levels of SBP and MAP, as well as lower risk of hypertension. This indicates a potential role of CLCN6 in BP regulation and positions this gene as an attractive therapeutic target for future studies.

We demonstrated that the effect of CLCN6 was independent of a previously reported common GWAS SNP in this region. CLCN6 is located in 1p36, a region with several BP candidate genes identified by GWAS, including AGTRAP,
Table 1. T1 Gene-Based Results for CLCN6 on 4 BP Traits Across 2 Ancestries in Cohorts for Heart and Aging Research in Genomic Epidemiology and Exome Sequencing Project

| Cohort          | Gene    | cMAF | n     | P Value | β (SE)    | P Value |
|-----------------|---------|------|-------|---------|-----------|---------|
| European ancestry |         |      |       |         |           |         |
| ARIC            | NPPA    | 0.01 | 5704  | 0.002   | −2.88 (0.95) | 0.002   |
| CHS             | NPPA    | 0.01 | 683   | 0.22    | −4.10 (3.31) | 0.29    |
| FHS             | NPPA    | 0.01 | 1227  | 0.03    | −3.92 (1.77) | 0.04    |
| Blacks          |         |      |       |         |           |         |
| ARIC            | NPPA    | 0.007| 2792  | 0.05    | −4.00 (2.07) | 0.09    |
| Multietnic      |         |      |       |         |           |         |
| CHARGE          | NPPA    | 0.01 | 10406 | 1.39×10⁻⁵ | −3.28 (0.76) | 0.025   |

Table 2. T1 Gene-Based Results for CLCN6 on Diastolic Blood Pressure in CHARGE Conditioning on rs17367504

| Cohort          | Gene    | cMAF | n     | P Value | β (SE)    | P Value |
|-----------------|---------|------|-------|---------|-----------|---------|
| European ancestry |         |      |       |         |           |         |
| ARIC            | NPPA    | 0.01 | 5704  | 0.002   | −2.88 (0.95) | 0.002   |
| CHS             | NPPA    | 0.01 | 683   | 0.22    | −4.10 (3.31) | 0.29    |
| FHS             | NPPA    | 0.01 | 1227  | 0.03    | −3.92 (1.77) | 0.04    |
| Blacks          |         |      |       |         |           |         |
| ARIC            | NPPA    | 0.007| 2792  | 0.05    | −4.00 (2.07) | 0.09    |
| Multietnic      |         |      |       |         |           |         |
| CHARGE          | NPPA    | 0.01 | 10406 | 1.39×10⁻⁵ | −3.28 (0.76) | 0.025   |

β corresponds to mm Hg per mutated allele for BP traits. cMAF indicates cumulative minor allele frequency; DBP, diastolic blood pressure; EA, European ancestry; MAP, mean arterial pressure; PP, pulse pressure; and SBP, systolic blood pressure.
baseline, and repeat measurements may provide a more precisely estimated phenotype to detect genetic determinants for BP variation.\textsuperscript{43} Therefore, future WES studies incorporating repeated BP measurements are justified.

In summary, by analyzing WES, we identified that an aggregation of rare coding variants in \textit{CLCN6} was associated with lower DBP and lower risk of hypertension among 13,409 EAs and 4,547 blacks from 8 large population-based cohort studies. In addition, the effect sizes of \textit{CLCN6} were consistent across two ancestries. Our findings provide evidence for a functional role of \textit{CLCN6} in BP regulation and point toward this gene as a therapeutic target.

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Figure 2. Cohort and ancestry-specific effects of \textit{CLCN6} on diastolic blood pressure (DBP) in 2-stage discovery cohorts. $\beta$ corresponds to mm Hg per mutated allele for DBP. ARIC indicates Atherosclerosis Risk in Communities; CHS, Cardiovascular Health Study; cMAF, cumulative minor allele frequency; EA, European ancestry; ESP, Exome Sequencing Project; FHS, Framingham Heart Study.

| Study | N | cMAF | Beta | SE | P |
|-------|---|------|------|----|---|
| Discovery I | | | | | |
| ARIC_EA | 5704 | 0.011-2.88 | 0.95 | 2.0e-03 | |
| CHS_EA | 5800 | -0.10-4.10 | 3.31 | 2.2e-06 | |
| FHS_EA | 1227 | 0.011-3.52 | 1.77 | 3.0e-02 | |
| ESP_EA | 2339 | 0.008-3.42 | 2.43 | 1.6e-01 | |
| ARIC_black | 2792 | 0.007-4.00 | 2.07 | 5.0e-02 | |
| ESP_black | 1755 | 0.011-3.04 | 2.26 | 1.8e-01 | |
| Discovery II | | | | | |
| RS_EA | 2205 | 0.004-1.19 | 2.30 | 6.8e-01 | |
| ERF_EA | 1254 | 0.003-7.26 | 3.60 | 4.0e-02 | |
| Two-stage discovery meta-analysis | 17956 | 0.011-3.30 | 0.66 | 5.0e-07 |
Disclosures

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Appendix

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CLINICAL PERSPECTIVE

Genetic variants that are rare in the general population may influence blood pressure. Our study focused on the protein coding (exome) sequence from 17,956 individuals of European ancestry and African ancestry (14,497, first-stage discovery and 3,459, second-stage discovery) and identified rare coding variants in \textit{CLCN6} significantly associated with lower diastolic blood pressure. The association persisted after conditioning on a nearby known blood pressure–related common variant, rs17367504. \textit{CLCN6} was also shown to have effects on other blood pressure traits, including systolic blood pressure and mean arterial pressure, and decreased odds of hypertension. \textit{CLCN6} belongs to the voltage-dependent chloride channel family with a known domain that is involved in blood pressure regulation. Corroborating evidence comes from a separate study showing that a knockout homologue \textit{Clcn6} in the rat reduced blood pressure levels and lowered hypertension risk. Our study showed that \textit{CLCN6} rare coding variants have a similar magnitude of effect on blood pressure levels and hypertension compared with common variants reported by genome-wide association studies, and the effect was consistent between European ancestry and African ancestry. These findings implicate the roles of rare coding variants in explaining blood pressure variation, contributing to hypertension, and suggesting potential therapeutic interventions for cardiovascular diseases.