The Role of Rare Variants in Systolic Blood Pressure: Analysis of ExomeChip Data in HyperGEN African Americans

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Key Words

Rare variants · ExomeChip · Gene-based analysis · Burden tests · SKAT · Systolic blood pressure · Family studies

Abstract

Cardiovascular diseases are among the most significant health problems in the United States today, with their major risk factor, hypertension, disproportionately affecting African Americans (AAs). Although GWAS have identified dozens of common variants associated with blood pressure (BP) and hypertension in European Americans, these variants collectively explain <2.5% of BP variance, and most of the genetic variants remain yet to be identified. Here, we report the results from rare-variant analysis of systolic BP using 94,595 rare and low-frequency variants (minor allele frequency, MAF, <5%) from the Illumina exome array genotyped in 2,045 HyperGEN AAs. In addition to single-variant analysis, 4 gene-level association tests were used for analysis: burden and family-based SKAT tests using MAF cutoffs of 1 and 5%. The gene-based methods often provided lower p values than the single-variant approach. Some consistency was ob-

served across these 4 gene-based analysis options. While neither the gene-based analyses nor the single-variant analysis produced genome-wide significant results, the top signals, which had supporting evidence from multiple gene-based methods, were of borderline significance. Though additional molecular validations are required, 6 of the 16 most promising genes are biologically plausible with physiological connections to BP regulation.

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Introduction

Cardiovascular diseases are among the most significant health problems in the United States today, with hypertension affecting African Americans (AAs) disproportionately. Blood pressure (BP) and hypertension have been challenging phenotypes for gene discovery due to their complex nature. Although GWAS have successfully identified thousands of loci affecting complex human diseases, these identified loci only explain small proportions of heritability. For example, 3 recent GWAS consortia

[1–3] identified dozens of common variants associated with BP and hypertension in European Americans. However, these variants collectively explain <2.5% of BP variance, and most of the genetic variants remain yet to be identified [4]. The missing heritability might be partially explained by rare and low-frequency variants, with minor allele frequencies (MAF) <5% [5].

Because the power to detect individual rare variants is limited by their low frequency, the development of novel statistical approaches for the analysis of rare variants has been an active area of research. These methods are broadly classified as burden and non-burden tests. Burden tests collapse rare variants in a genomic region (typically a gene) into a single burden variable to test for the combined effects of all rare variants in the region or gene. Such tests include the cohort allelic sum test (CAST) [6], the combined multivariate and collapsing (CMC) method [7], the group-wise weighted sum test [8], and the proportion and presence tests by Morris and Zeggini [9]. Since these burden tests implicitly assume that all variants influence the trait in the same direction and with the same magnitude of effect, their power is reduced if their effects are not homogeneous [10]. Several non-burden tests have been developed that are less sensitive to heterogeneous effects [11–13]. Two one-sided statistics proposed by Ionita-Laza et al. [11] quantify enrichment in risk variants and protective variants. The C-alpha test uses the distribution of genetic variation observed in cases and controls, maintaining consistent power when the target set contains both risk and protective variants [12]. The sequence kernel association test (SKAT) provides a flexible and computationally efficient approach using a score-based variance component test and is applicable to both continuous and discrete traits [13]. Using the framework of linear mixed effect models, SKAT has been recently extended to handle family data, which is called the family-based SKAT (famSKAT) [14].

Here, we report the results from rare-variant analysis using 94,595 rare and low-frequency variants from the Illumina exome array (ExomeChip) genotyped in HyperGEN AAs. We performed 4 gene-level association analyses of systolic BP (SBP): burden and famSKAT tests with MAF cutoffs of 1 and 5% accounting for family correlation. To account for population stratification in our AA subjects, we used principal components (PCs) of the common variants (with MAF $\geq 5\%$) on the ExomeChip as covariates in our analysis. A varying number of PCs (no PC, first 2 PCs and first 10 PCs) were used to evaluate the impact of population stratification on the analysis of rare variants.

Methods

Study Sample

We studied AAs in the Hypertension Genetic Epidemiology Network (HyperGEN) from the Family Blood Pressure Program [15]. The study recruited AA and European American participants at 5 field centers to investigate the genetic causes of hypertension and related conditions. Study participants were 1 of 3 types: (1) individuals in hypertensive sibships with at least 2 siblings diagnosed with hypertension; (2) random subjects, who were age-matched with hypertensive siblings, or (3) unmedicated adult offspring of 1 or more of the hypertensive siblings. The study obtained informed consent from all participants and approval from the appropriate institutional review boards.

Genotype and Phenotype Data

A total of 2,147 HyperGEN AAs were genotyped using the Illumina exome array that covers the genome-wide exome variants. Illumina's clustering algorithm (GenTrain version 1.0), as implemented in GenomeStudio, was used for genotype calling. To check the quality and consistency of the genotyping process, 19 blind duplicate samples were included. We removed these duplicate samples and corrected sample mix-ups and pedigree errors, resulting in 2,111 subjects. We removed monomorphic markers and SNPs with a missing rate >5% or Hardy-Weinberg p value <10⁻⁶ and removed any genotypes with a non-Mendelian pattern of inheritance. The number of variants after quality control and exclusions was 124,759. There were 30,164 common variants (with MAF $\geq 5\%$) and 94,595 low-frequency variants (with MAF <5%). There were also extremely rare variants including 15,017 singletons and 9,323 doubletons.

We included 2,045 subjects with genotype, phenotype and covariate information. SBP was measured (on a mm Hg scale) with a DINAMAP model 1846 SX/P automated oscillometric device using a standard protocol. Briefly, after a 5-min rest period, the resting BP was measured 6 times, with a 2-min gap between the measurements. The average of 3 SBP measurements was used for each subject. In all analyses, we added 15 mm Hg to the SBP values for subjects taking antihypertensive or BP-lowering medications. Age, sex, and body mass index were included as covariates. In addition, a varying number of PCs derived from common variants were included as covariates, as discussed below.

Statistical Analysis

We performed a single-variant analysis and the 4 sets of genome-wide gene-level association analysis. The latter are burden tests with MAF cutoffs of 1 and 5% (Burden-1 and Burden-5) and famSKAT tests with MAF cutoffs of 1 and 5% (famSKAT-1 and famSKAT-5). We used the annotation file for the Illumina exome array data, available from the CHARGE (Cohorts for Heart and Aging Research in Genetic Epidemiology) study [16]. For the single-variant analysis, we excluded singletons because false positive results may arise from an influential phenotype value of one subject. For the gene-level analyses, we excluded genes with only one variant as their results were identical to those of the single-variant analysis. We included singletons for the gene-level analyses. For example, when a gene included multiple singletons, all these singletons were jointly used for our gene-based analyses.

To perform the burden tests in HyperGEN AAs, we extended the burden test by Morris and Zeggini [9] to account for family

Table 1. Genomic inflation values (λ) for the 5 genome-wide analysis options using each of PC adjustments

PCs	Single-variant analysis	Burden-1	Burden-5	famSKAT-1	famSKAT-5
0	1.002	1.028	1.007	1.075	1.094
2	0.999	1.025	1.006	1.079	1.102
10	0.999	1.041	1.010	1.107	1.119

Quantile-quantile plots are shown in online supplementary figure 1.

Table 2. The 11 top low-frequency variants from the single-variant analysis with a p value $< 6 \times 10^{-5}$ (all these variants were exonic nonsynonymous)

Rank	SNP	Chr	BP	Gene	MAF	Beta	SE	p
1	rs138085317	4	114,290,810	ANK2	0.0042	27.3	5.6	1.2×10^{-6}
2	rs143104022	18	30,791,983	<i>C18orf34</i>	0.0012	43.3	9.9	1.2×10^{-5}
3	rs144164391	6	25,779,375	<i>SLC17A4</i>	0.0012	42.8	9.9	1.4×10^{-5}
4	rs75307540	11	6,452,889	HPX	0.0093	15.9	3.8	2.9×10^{-5}
5	rs145926130	16	1,536,575	<i>PTX4</i>	0.0017	35.8	8.6	3.2×10^{-5}
6	rs35124934	2	3,744,990	ALLC	0.0342	8.1	2.0	4.3×10^{-5}
7	rs6665210	1	145,532,823	<i>ITGA10</i>	0.0193	-10.7	2.6	4.4×10^{-5}
8	rs75508009	5	111,519,758	<i>EPB41L4A</i>	0.0022	30.2	7.4	4.5×10^{-5}
9	rs144714540	1	45,809,001	<i>TOE1</i>	0.0010	48.8	12	4.6×10^{-5}
10	rs139543898	1	158,389,863	<i>OR10K2</i>	0.0005	63.4	15.6	5.0×10^{-5}
11	rs146171474	4	122,833,191	TRPC3	0.0007	56	13.9	5.8×10^{-5}

The genome-wide Bonferroni-corrected threshold corresponds to a p value $< 6.4 \times 10^{-7}$ ($=0.05/79,578$). Bold-faced genes are biologically plausible, providing a physiological connection to BP regulation (as presented in the Discussion section). Except for *TRPC3*, which contained only 1 variant, these were included in table 3.

structure. For the combined effect of multiple rare variants in a genomic region, Morris and Zeggini [9] developed the burden test in a linear regression framework. For family data, a linear mixed model with a random polygenic component is commonly used to account for phenotypic correlations among related individuals. Therefore, we used a linear mixed effect modeling framework and performed our analysis with GenABEL [17] and ProbABEL [18], which uses the kinship matrix based on the pedigree structure.

Population stratification is known to create spurious associations if not properly controlled. The common practice in GWAS has been to control for stratification by using a number of top PCs of the GWAS markers as covariates in the association analysis. However, the optimal number of PCs has not been well established even for common-variant analysis, as shown by Peloso and Lunetta [19]. Therefore, to account for population stratification in our AA subjects, we used a varying number of the leading PCs as covariates in our analysis: no PC, the top 2 PCs and the top 10 PCs. Although the impact of population stratification on the analysis of rare variants has shown to be more subtle and complex [20–23], several papers have demonstrated advantages of using PCs derived from common variants (with MAF $\geq 5\%$) [24–27]. Therefore, we computed PCs derived from 30,164 common variants in our

ExomeChip data. The genomic inflation factor λ was calculated as the ratio of the observed median χ^2 value to the expected median χ^2 value.

Results

We performed a single-variant analysis and the 4 sets of genome-wide gene-based analysis using 79,578 low-frequency variants: Burden-1, Burden-5, famSKAT-1, and famSKAT-5. For a varying number of PCs, the genomic inflation factors λ are shown in table 1. The single-variant analysis exhibited no genomic inflation, whether PCs were included or not (with all $\lambda \leq 1.002$). Likewise, the burden tests did not exhibit much inflation, with 2 PCs leading to the lowest inflation ($\lambda = 1.025$ and 1.006 for Burden-1 and Burden-5, respectively). In contrast, the SKAT analyses exhibited modest inflation, with no PCs leading to the lowest inflation ($\lambda = 1.075$ and 1.094 for

Fig. 1. Manhattan plots of the single-variant analysis and the 4 gene-level analysis options (Burden-1, Burden-5, famSKAT-1, and famSKAT-5). For the single-variant analysis, the $-\log_{10}(p)$ of each SNP was plotted at the chromosomal location of the SNP. For each

gene-level analysis, the $-\log_{10}(p)$ of each gene was plotted at the chromosomal location of the first SNP of the gene. These results are based on using 2 PCs.

famSKAT-1 and famSKAT-5, respectively). Based on table 1 and also because our subjects were of African ancestry, we decided to present the results using 2 PCs. Quantile-quantile plots are shown in online supplementary figure 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000375373).

Manhattan plots for the single-variant analysis and the 4 sets of gene-based analysis using 2 PCs are shown in figure 1. The genome-wide Bonferroni-corrected threshold was 6.4×10^{-7} ($=0.05/79,578$) for the single-variant analysis. The gene-based tests with MAF cutoffs

of 1 and 5% included 11,958 and 12,895 genes, respectively; the corresponding Bonferroni-corrected thresholds were 4.2×10^{-6} and 3.9×10^{-6} , respectively. Table 2 presents the top 11 low-frequency variants from our single-variant analysis (with $p < 6.0 \times 10^{-5}$). Table 3 shows the top 16 genes from the 4 gene-based results (with $p < 2.0 \times 10^{-4}$). We found no genome-wide significant results based on the Bonferroni-corrected threshold. However, we found that the gene-based methods often provided lower p values than the single-variant approach, as shown in table 3.

We found that all 16 top genes in table 3 indeed had supporting evidence from multiple gene-based methods. To further evaluate the relative performance of each gene-based method, we classified the genes in table 3 into 4 groups. Note that the low-frequency variants (with $1\% \leq \text{MAF} < 5\%$) are presented in parenthesis in the ‘Direction of single-variant association’ column. For genes in group 1, the Burden-1 approach provided the strongest evidence; at these genes, rare variants had mostly unidirectional effects. We found that famSKAT also provided corroborating evidence for some of those genes (notably *NSUN5* and *FGF4*), which is assuring. When the low-frequency variants had the opposite effects as compared to rare variants (as for *OR52H1*), the Burden-5 approach provided much weaker evidence than Burden-1. In contrast, when the low-frequency variants had the same effects as rare variants, the Burden-5 approach provided stronger evidence than Burden-1, as shown in group 2. We found that famSKAT indeed performed better on genes with multidirectional variants, as shown in groups 3 and 4 (notably *HPX*).

To improve the selection of variants to be included in the gene-level tests, we also considered functional annotation information. It turned out that most (90,875 out of

Group	Gene	Gene position ^a	Gene-level association p values			Direction of single-variant association ^b	Top single-variant signal (MAF <5%)			
			Burden-1	Burden-5	famSKAT-1		famSKAT-5	SNP ^c	MAF	p value
1	DOPEY2 SUN5 FGF4 ORS2H1 ANKRD50 ADC RAB3GAP1	Chr 21: 37.5 Mb	8.3×10 ⁻⁵	2.8×10 ⁻³	8.1×10 ⁻⁴	2.6×10 ⁻³	+++++-----+(-)-----+++	rs142091518	0.0002	6.1×10 ⁻⁴
		Chr 7: 72.7 Mb	8.6×10 ⁻⁵	8.6×10 ⁻⁵	3.6×10 ⁻³	3.6×10 ⁻³	++++	rs77133388	0.0002	6.5×10 ⁻³
		Chr 11: 69.6 Mb	1.2×10 ⁻⁴	1.2×10 ⁻⁴	5.6×10 ⁻⁴	5.6×10 ⁻⁴	++	rs140567674	0.0002	2.9×10 ⁻³
		Chr 11: 5.6 Mb	1.2×10 ⁻⁴	0.73	5.5×10 ⁻⁴	0.27	++(+)(+)(-)(-)+---(+)(+)+	rs143228643	0.0037	4.0×10 ⁻⁴
		Chr 4: 125.6 Mb	1.5×10 ⁻⁴	0.77	1.6×10 ⁻⁴	0.55	(-)(+)(-)	rs140645873	0.0002	1.5×10 ⁻⁴
		Chr 1: 33.6 Mb	1.5×10 ⁻⁴	0.17	6.2×10 ⁻³	8.6×10 ⁻³	+++++-----	rs150169064	0.0044	0.02
		Chr 2: 135.8 Mb	1.7×10 ⁻⁴	0.13	5.1×10 ⁻³	0.03	(-)(++++)(+)(-)(-)-	rs150478342	0.0017	1.7×10 ⁻⁴
2	FANK1 ANKRD6	Chr 10: 127.7 Mb	0.05	1.7×10 ⁻⁴	0.51	9.3×10 ⁻³	---(-)(-)-(-)---	rs141545587*	0.0005	0.01
		Chr 6: 90.3 Mb	0.26	1.7×10 ⁻⁴	0.51	4.3×10 ⁻³	(+)(+)+	rs61736690*	0.0472	7.3×10 ⁻³
3	HPX MARCH8 ARHGAP1 CD3E	Chr 11: 6.5 Mb	3.2×10 ⁻⁴	0.41	7.1×10 ⁻⁵	4.0×10 ⁻³	+++++(-)(-)(-)(-)	rs75307540	0.0093	2.9×10 ⁻⁵
		Chr 10: 46.0 Mb	0.01	0.01	1.2×10 ⁻⁴	1.2×10 ⁻⁴	---	rs140989641	0.0022	1.0×10 ⁻⁴
		Chr 11: 46.7 Mb	7.5×10 ⁻³	7.5×10 ⁻³	1.4×10 ⁻⁴	1.4×10 ⁻⁴	-+-	rs11822837*	0.0044	1.5×10 ⁻⁴
		Chr 11: 118.2 Mb	4.6×10 ⁻⁴	4.6×10 ⁻⁴	1.5×10 ⁻⁴	1.5×10 ⁻⁴	+++	rs140639753	0.0015	1.1×10 ⁻⁴
		Chr 6: 39.9 Mb	0.11	2.3×10 ⁻⁵	0.01	1.4×10 ⁻⁵	+-+(-)++-	rs7762875	0.0208	6.2×10 ⁻⁵
4	MOC1 ITGA10	Chr 2: 3.7 Mb	0.95	1.5×10 ⁻⁴	0.03	1.7×10 ⁻⁵	-----+(+)(+)(+)	rs5124934	0.0342	4.3×10 ⁻⁵
		Chr 1: 145.5 Mb	0.15	8.8×10 ⁻³	1.3×10 ⁻³	8.6×10 ⁻⁵	-+(-)+-+(-)+-----+(-)(+)(+)(-)-	rs6665210	0.0193	4.4×10 ⁻⁵

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94,595) low-frequency variants were nonsynonymous, stop-gain/loss, splice, or missense variants. Therefore, when we repeated all 4 gene-level analyses with these functional variants only, the results were very similar (Spearman correlation = 0.97) as shown in the first row of online supplementary figure 3. Roughly half ($n = 41,393$) of the low-frequency variants were stop-gain/loss, splice or missense variants and predicted to be damaging by 2 of the 4 algorithms in dbNSFP (Mutation Taster, Polyphen HDIV, SIFT or LRT). We further performed the 4 gene-level analyses with these damaging variants and found a subset of genes with lower p values (shown in the second row of online suppl. fig. 3). The most notable one was *MOCS1*, where the Burden-5 result was enhanced ($p = 4.5 \times 10^{-6}$). Our results illustrate that excluding neutral variants may help to improve the power of gene-level tests.

Although our focus was on low-frequency variants, we also performed a single-variant analysis on common variants. Among 30,164 common variants, our strongest signal was observed at rs11711441 on chromosome 3 (with $p = 1.7 \times 10^{-5}$). Out of 60 known BP variants identified by 9 articles [3, 28–35], only 13 were available in our ExomeChip data. Among these 13 known variants, the smallest p value from our analysis was observed at rs3184504 in *SB2B3* (with $p = 0.0029$). The effect had the same direction as that reported by the International Consortium for Blood Pressure Genome-Wide Association Studies [3], but with lower MAF (0.057). Our results may not be a surprise given the limited number of common variants in the ExomeChip data.

Discussion

We performed an analysis of SBP using 79,578 rare and low-frequency variants from the Illumina exome array genotyped in 2,045 HyperGEN AAs. While neither the gene-based analyses nor the single-variant analysis produced genome-wide significant results, the most promising genes from each of the 5 methods had supporting evidence from some of the other gene-based methods. Of the genes for which the burden approach provided strongest evidence, 3 genes are of particular interest as potential BP candidate genes: *NSUN5* (NOP2/Sun domain family), *ADC* (arginine decarboxylase) and *RAB3GAP1* (RAB3 GTPase-activating protein subunit 1). All 3 genes had corroborating evidence from SKAT as well. As the burden approach provided more significant evidence, the SNP effects in these genes are likely ho-

mogeneous. *NSUN5* is shown to be deleted in Williams-Beuren syndrome [36], a multi-system developmental disorder whose symptoms include hypertension [37]. *ADC* contains a conformational change-inducing missense mutation (substituting a polar amino acid, serine, for a nonpolar one, alanine) that has been previously associated with SBP [38]. *RAB3GAP1* has been previously identified to be associated with sudden cardiac death in the MetaboChip study [39].

Of the genes with strong evidence from famSKAT analysis, 3 have potential connections to BP: *HPX* (Hemopexin), *ARHGAP1* (Rho GTPase-activating protein 1) and *ALLC* (allantoicase). *HPX* is a plasma glycoprotein that transports heme to the liver, and it is a major BP candidate gene owing to its role in regulating the expression of the angiotensin II type I receptor *AGTR1* and modulating sensitivity to angiotensin [40] as well as its identification as a risk factor for late renal graft failure [41]. *ARHGAP1* is a guanosine-triphosphate-metabolizing enzyme that plays a role in the regulation of tubule formation, an important early step in angiogenesis. *ALLC* is involved in the uric acid degradation pathway; uric acid levels have been associated with BP [42].

The single-variant analysis provided evidence for 2 additional potential candidate genes: *ANK2* (ankyrin 2) and *TRPC3* (transient receptor potential cation channel). *ANK2* is a member of the ankyrin family of proteins and is required for the targeting and stability of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger in cardiomyocytes. Mutations in this gene lead to long-QT syndrome and cardiac arrhythmia [43]. *ANK2* has been located in a 1-lod support interval linked to resting heart rate [44]. *TRPC3* is a renal Ca^{2+} channel, expressed in principal cells of the collecting duct. The expression levels of *TRPC3* have been associated with systolic hypertension in humans [45] and are higher in spontaneously hypertensive rats than in normotensive Wistar-Kyoto rats [46].

To evaluate the impact of population stratification on the analysis of rare variants, we used a varying number of leading PCs as covariates in our analysis. We found that type I error rates were well controlled for the single-variant and burden approaches, whereas the famSKAT approach exhibited modest inflation, as also noted by the famSKAT developers [14]. Furthermore, a recent article by Zawistowski et al. [23] also reports higher levels of inflation from the other non-burden tests including the C-alpha test [12] and the original SKAT [13]. These non-burden tests have greater power to detect association in genes containing rare variants with heterogeneous effects

but may be more vulnerable to population stratification. Given the lack of significant findings due to low power to detect rare variants, consistent findings from multiple gene-based methods including both burden and non-burden approaches with different thresholds are reassuring. These findings are compelling candidates for external validation.

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