Multiple Genes for Essential-Hypertension Susceptibility on Chromosome 1q

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Essential hypertension, defined as elevated levels of blood pressure (BP) without any obvious cause, is a major risk factor for coronary heart disease, stroke, and renal disease. BP levels and susceptibility to development of essential hypertension are partially determined by genetic factors that are poorly understood. Similar to other efforts to understand complex, non-Mendelian phenotypes, genetic dissection of hypertension-related traits employs genomewide linkage analyses of families and association studies of patient cohorts, to uncover rare and common disease alleles, respectively. Family-based mapping studies of elevated BP cover the large intermediate ground for identification of genes with common variants of significant effect. Our genomewide linkage and candidate-gene–based association studies demonstrate that a replicated linkage peak for BP regulation on human chromosome 1q, homologous to mouse and rat quantitative trait loci for BP, contains at least three genes associated with BP levels in multiple samples: *ATP1B1, RGS5,* and *SELE*. Individual variants in these three genes account for 2–5-mm Hg differences in mean systolic BP levels, and the cumulative effect reaches 8–10 mm Hg. Because the associated alleles in these genes are relatively common (frequency >5%), these three genes are important contributors to elevated BP in the population at large.

Essential hypertension (EH [MIM 145500]), defined as chronically elevated blood pressure (BP) occurring in the absence of other predisposing conditions, affects >25% of adults worldwide and is a significant risk factor for coronary heart disease, stroke, and renal disease.[1] Genes play a major role in determination of BP and EH susceptibility, because the heritability of BP levels has been estimated to be 30%–35%.[2] Rare syndromic forms of hyper- and hypotension show Mendelian inheritance, and the mutations underlying some of these disorders have been identified by positional cloning and candidate-gene analyses.[3,4] These genes all regulate renal salt reabsorption, in accordance with earlier work by Guyton[5] and others that established that the kidney plays a central role in BP regulation. Mutations underlying monogenic hypertension are rare in the general population; thus, the genetic basis of EH remains poorly understood, and numerous loci segregating alleles of smaller effect are still unidentified.

More than 30 genomewide linkage studies of EH and BP-related phenotypes have been published (reviewed by Garcia et al.[6] and Samani[7]), by use of a variety of study designs and populations. Meta-analyses of these studies indicate that there is no single genomic region with a large effect on BP or EH. However, many linkage studies were insufficiently powered to detect small effects. Nevertheless, several genomic regions, such as 1q, 2p, 3p, 6q, 7q, 12q, 15q, 17q, 18q, and 19p, were validated by multiple studies and are likely to contain EH-susceptibility loci.[7] These linkage regions, typical of complex traits, contain hundreds of genes. Therefore, it is difficult to decide which linkage regions warrant costly gene identification.

We report the characterization of a linkage region on chromosome 1q23-q32 that is validated in several BP and EH linkage studies and is supported by BP-related QTL mapping studies in the rat and mouse. To optimize power, we started with samples collected through family-based studies enriched with subjects with elevated BP unaltered by medication. We also vigorously rid our pedigree structure, phenotype, and genotype, and marker data of all detectable errors. Although the genetic basis of hypertension is largely unknown, physiological processes that regulate and alter BP are better understood. Therefore, we took advantage of the published literature and annotation of known genes in our linkage region, and we focused on genes that are in pathways that determine BP levels or have a regulatory function. We found evidence of association between three genes and BP in multiple samples. The genes are independently associated with BP levels, and the cumulative effects of having multiple susceptibility alleles at these three genes are estimated to be 8–10 mm Hg. Our results indicate that the 1q locus harbors multiple EH-susceptibility genes that affect BP levels.

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Material and Methods

Study Population

Samples used in this study were collected through three networks of the NHLBI Family Blood Pressure Program (FBPP): GenNet, GENOA, and HyperGEN. Details of the FBPP subjects have been published elsewhere,[8] and characteristics of GenNet, GENOA, and HyperGEN samples are provided in table 1. GenNet samples were used in the original linkage and association studies and consist of 1,826 individuals from 592 families that are either European American (EA) or African American (AA), collected from 1995 to 2000. Probands were subjects between the ages of 18 and 50 years with BP in the upper 25% of the age- and sex-specific BP distribution. Overall, 33% of the study subjects were clinically hypertensive, with systolic BP (SBP) ⩾140 or diastolic BP (DBP) ⩾90 mm Hg, or were taking hypertension medication at the time of the study. The 592 families consist of bigenerational pedigrees and sibships. Most (91%) of the GenNet AA families consisted of siblings only, whereas 24%, 29%, and 47% of GenNet EA families included genotype and phenotype information from both, one, and no parents, respectively. Two other FBPP networks, GENOA and HyperGEN, provided EA and AA samples, to confirm the association between chromosome 1 variants and BP (2,944 and 1,135 samples from GENOA and HyperGEN, respectively). Probands were those with either severe or familial hypertension. On the basis of the same definition as above, 69% and 39% of the GENOA and HyperGEN subjects, respectively, are clinically hypertensive. This study was approved by the institutional review boards at the parent institutions of all participating FBPP laboratories.

Phenotype

BP measurement and the inclusion and exclusion criteria for GenNet, GENOA, and HyperGEN samples have been described elsewhere.[8–11] For example, two manual BP readings were taken from GenNet subjects with the use of a standard mercury manometer. The average SBP and DBP readings were used for analysis. Pulse pressure (PP) was defined as SBP minus DBP. BMI was calculated as weight in kilograms divided by height in meters squared. To avoid likely erroneous extreme BP values, all SBP reading >180 mm Hg or <85 mm Hg and all DBP readings >110 mm Hg or <40 mm Hg were used in the analysis only if the difference between the first and second readings was <10 mm Hg. For some analyses, BP values for the subjects taking hypertension medication were adjusted for an average treatment effect by addition of 10 mm Hg to SBP and 5 mm Hg to DBP, as suggested elsewhere.[12]

Genotyping and Data Quality Control

Microsatellite markers used in genomewide linkage scans were genotyped by the Mammalian Genotyping Service from 1998 to 2000, by use of five sets of markers. The average intermarker distance was 9.2 cM. Data from the 22 autosomes were analyzed and presented. To further characterize the 1q linkage region, 16 additional microsatellite markers in 1q23.1-32.1 were genotyped using the deCODE genotyping service. After fine mapping, the average intermarker distance in the 1q region was reduced from 8.2 Mb to <1.9 Mb.

The microsatellite genotypes and family data of GenNet underwent an extensive data-cleaning process that involved (1) identification and deletion of markers in which alleles were called inconsistently among the seven batches of genotypes generated, (2) validation and correction of family structures on the basis of the mean and SD of allele sharing (by use of microsatellite genotype data) of all pairs of individuals within the study, and (3) removal of all other probable genotyping errors that are inconsistent with Mendelian transmission. Overall, 10% of the genotypes were unavailable because of genotyping failure or deletion of erroneous data. Our data-cleaning protocol, developed on the basis of our experience with marker and family data for 12,041 FBPP subjects, and examples of the importance data quality can have for the outcome of genetic analyses are published elsewhere.[13]

SNP genotyping of 1q candidate genes was done using 5′-nuclease–based assay (TaqMan [ABI]). Data quality of SNP genotypes was established by three methods: reproducibility of control DNA samples, expected Mendelian inheritance of alleles within a family (PedCheck[14]), and Hardy-Weinberg equilibrium tests ($P > .05$). For the last, we used all unrelated individuals in our study, analyzing samples from different ethnic groups separately, using the analysis package Haploview.[15]

Candidate-Gene Selection

Given our incomplete understanding of BP regulation, a systematic search for variants associated with BP should be conducted for all genes in the linkage region, and this effort is underway.

Table 1. Samples Studied

	Original Sample GenNet		Replication Sample			
			GENOA		HyperGEN	
Characteristic	EA	AA	EA	AA	EA	AA
No. of subjects	1,010	816	1,324	1,620	810	325
No. of families	303	289	473	559	272	78
Mean age (years)	45.6 ± 14.8	41.3 ± 11.7	55.4 ± 11.1	57.4 ± 10.3	53.5 ± 13.3	42.0 ± 13.3
Male (%)	45	40	46	32	45	35
Mean SBP (mm Hg)	123.2 ± 17.6	123.9 ± 19.7	134.0 ± 19.9	130.7 ± 23.3	116.9 ± 20.5	123.2 ± 22.8
Mean DBP (mm Hg)	77.0 ± 10.0	76.0 ± 13.4	76.2 ± 10.3	70.5 ± 11.5	66.8 ± 10.1	70.9 ± 12.3
Mean BMI	29.0 ± 6.1	30.4 ± 8.4	30.4 ± 6.3	30.9 ± 6.6	28.4 ± 5.9	30.9 ± 7.4
Normotensive (%)	68	66	33	29	61	62
EH (%)[a]:						
Medicated	22	22	65	61	37	32
Unmedicated	10	12	2	10	2	6

[a] Medicated = hypertensive patients taking hypertension medication; Unmedicated = hypertensive individuals not taking hypertension medication.

During the initial round of candidate-gene selection, 35 genes were chosen on the basis of their known or putative functions, and they are listed in table 2. These protein products play roles in salt transport, signal transduction, inflammatory response, renal filtration, lipid metabolism, and vascular function—pathways thought to be dysregulated in hypertension. They also included regulators and signal transduction proteins with little or no known function related to BP regulation. From this list, nine genes were selected for SNP analysis (fig. 1). Eight genes were selected on the basis of their physical proximity to the markers with the maximum LOD (MLOD) score in the original linkage scan and the two new peaks after fine mapping: *RGS4* (MIM 602515), *RGS5* (MIM 603276), *ATP1B1* (MIM 182330), *SELL* (MIM 153240), *SELE* (MIM 131210), *SELP* (MIM 173610), *REN* (MIM 179820), and *ADORA1* (MIM 102775). The distances between these genes and the linkage markers with MLOD scores ranged between 27 and 642 kb. In addition, we also took advantage of the mapping of BP-related loci in mice. By the combining of the linkage signals from two different crosses of hypertensive and normotensive strains (C57BL/6J × A/J and SWR/J × C3H/HeJ), the 95% CI of the mouse BP-related QTL on chromosome 1 was refined to an 18-cM region,[16] a region smaller than the homologous BP-related linkage region identified in the human and rat. Mouse homologs of six genes selected using our first criterion, *ATP1B1, SELE, SELP, SELL, ADORA1,* and *REN,* are located in this 18-cM region. The exceptions, *RGS4* and *RGS5,* are the candidate genes closest to the MLOD score peak of 4.3 (175.6 cM). High-density haplotype mapping of the same four mouse strains identified two regions (1.8 Mb and 2.5 Mb in size) that are shared by the hypertensive strains and are not shared by the normotensive strains.[16] Another gene from our list of 35 positional candidate genes, *NPHS2* (MIM 604766), is located in one of these two regions and was also selected.

SNP Selection

The 58 SNPs genotyped and analyzed were those validated by dbSNP or by our own sequencing of *ATP1B1, SELE,* and *RGS5*. All genotyped SNPs have minor-allele frequencies >0.05 in GenNet samples. The average distance between SNPs within a gene is 5.7 kb. To uncover novel variants associated with BP regulation in *RGS5, SELE,* and *ATP1B1,* we sequenced 100 unrelated GenNet EA samples by use of BigDye terminator system (ABI) with PCR and sequencing oligonucleotide primers designed using Primer3. For each of the three genes, all exons (and flanking intronic sequences) and conserved noncoding regions in the associated regions (FBAT $P < .05$) were sequenced.

Genetic Analysis

Genomewide linkage scan.—We applied nonparametric linkage analysis, using the variance-components approach implemented by analysis packages GENEHUNTER (version 2.1) and SOLAR (version 2.1). SBP, DBP, and PP were adjusted for age and sex, whereas other factors, such as BMI, smoking status, and diabetes, did not alter linkage signals significantly and were not included as covariates in the results presented. EA and AA samples were analyzed separately and in combination. Individuals taking antihypertensive medications at the time of the study either were analyzed with the use of the treated BP values or were excluded from analysis. Because the location and the magnitude of MLOD scores obtained by use of GENEHUNTER versus SOLAR were nearly identical (the MLOD score obtained for chromosome 1 was 4.9 at 176 cM vs. 4.6 at 175 cM, respectively), only results obtained from GENEHUNTER are presented in table 3 and figure 1.

Table 2. Hypertension Candidate Genes in 1q Linkage Region

The table is available in its entirety in the online edition of *The American Journal of Human Genetics.*

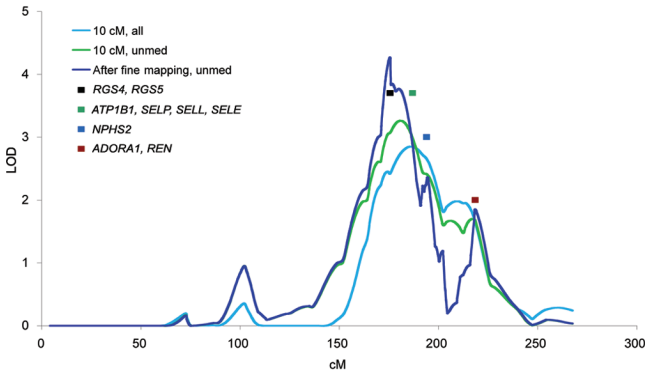

Figure 1. Genetic evidence for chromosome 1 linkage to DBP in GenNet EA samples. MLOD scores from an original linkage scan that analyzed all samples, regardless of medication status (10 cM, all), excluding those from patients taking hypertension medication (unmed), and with additional markers in the linked region (after fine mapping). The locations of the nine candidate genes are indicated (*colored squares*).

Comparison of human, rat, and mouse linkage results.—When comparing our linkage scan results with the published literature, we used rather stringent criteria to decide what constitutes a replicated linkage signal. Only linkage regions with MLOD score ⩾2 from each study were compared. Furthermore, the linkage result was considered replicated only when the markers closest to the MLODs of the two studies are identical. If different marker panels were used, replication was accepted only if the physical locations (based on National Center for Biotechnology Information [NCBI] build 34) of the markers closest to the MLODs in both studies are within the same cytogenetic subband, such as 1q24.

For comparison of our linkage results with rat BP-related QTL findings, we used the cytogenetic regions homologous to rat QTL clusters described by Stoll et al.[17] For mouse QTLs, when available,[16,18,19] we used the human cytogenetic regions homologous to rat and mouse QTLs stated in the literature. When the position was not well mapped,[20] we used the comparative genomic tools offered by the UCSC Genome Browser and the Comparative Maps Web site to locate the region in the human genome homologous to the mouse QTL.

Candidate-gene SNP association test.—Pairwise linkage disequilibrium (LD) between candidate-gene SNPs was calculated using Haploview,[15] and the LD-block definition was obtained from reference.[21] The software package FBAT[22] was used for family-based association tests for age- and sex-adjusted SBP and DBP. To test for possible haplotype effects, we used windows of two and three adjacent SNPs across the genes via haplotype FBAT (HBAT).[23] Associations with each of the 58 SNPs, as well as both global and haplotype-specific associations, were evaluated under the addi-

Table 3. Hypertension-Susceptibility Loci from Genomewide Linkage Mapping of GenNet Samples

Location	Position (cM)	Phenotype	Samples[a]	MLOD	Other Studies[b]	Animal Hypertension Models
1q25	182	DBP	EA	3.2	Family Heart Study[30]; Finnish Twin Cohort Study[28]; Framingham Heart Study[34]	Rat[17]; mouse[16,18]
2q33	200	PP	Combined	2.8	Amish Family Diabetes Study[31]	Mouse[20,33]
10p12	28	DBP	EA	2.6	Stanford Asian Pacific Program in Hypertension and Insulin Resistance[27]	…
10q21	86	DBP	Combined	2.6	International Collaborative Study on Hypertension[32]	…
14q13	41	PP	Combined	2.5	Finnish study[26]	…
14q32	106	PP	Combined	3.0	…	…
15q12	19	SBP	Combined	2.4	…	Mouse[33]
17q21	67	PP	Combined	2.2	Framingham Heart Study[29]	Rat[17]; mouse[33]

[a] Combined = EA and AA samples combined.
[b] Other hypertension linkage studies that provide genetic evidence of BP-related loci in the same genomic regions.

tive genetic model. The null hypothesis of no association in the presence of linkage was tested using the -e flag, which provides an empirical variance for the test statistic, and the -p flag, which provides Monte Carlo *P* values for the test statistics (up to 100,000 permutations). Since similar results were obtained, providing evidence of association with the same SNPs and haplotypes, only Monte Carlo *P* values calculated before correction for multiple testing are presented.

Effect-size estimation.—Population-based estimates of the effect of each SNP on age- and sex-adjusted SBP were made using general estimating equations (GEE) to account for familial correlation, as described elsewhere.[24] This method, available in the analysis package PBAT[25] (option 2), uses all available families (including uninformative families that do not contribute to the FBAT statistics) and is robust against population admixture and stratification.

Corrections for multiple testing.—Because we analyzed multiple SNPs that are in strong LD with each other and multiple phenotypes (such as SBP and DBP) that are also highly correlated, the individual FBAT tests performed were not truly independent. We attempted to correct for multiple testing without overly penalizing the tests. First, we determined the number of "independent" SNPs by counting SNPs in strong LD or an LD block as one (using the same criteria used by Gabriel et al.[21]) and SNPs outside of LD blocks individually. FBAT *P* values are corrected by the number of "independent" SNPs, analyzed using the Bonferroni method. Of the 58 SNPs analyzed in the GenNet EA samples, 31 are independent, and FBAT *P* values <.0016 are reported as significant after correction for multiple testing. In GenNet AA samples, there are 34 independent SNPs, and *P* values <.0011 are considered significant after correction. In our replication samples (GENOA and HyperGEN), only nine SNPs with prior association signals were analyzed. Because of their LD relationships, these nine SNPs constitute four independent tests, and replication association signals with $P < .0125$ are considered significant after correction for multiple testing.

Analysis of multiple independent phenotypes can also contribute to type I errors. However, the phenotypes analyzed in our study—SBP, DBP, and SBP/DBP adjusted for age, sex, medication, etc.—are highly correlated with one another. In our study, unadjusted SBP and DBP have R^2 of ~0.50, and no adjustment for testing of multiple phenotypes was made.

Results

Genomewide Linkage Scan of BP-Related QTLs

To gain insight into the genetic basis of EH, we applied variance-components linkage analysis to 1,010 European American (EA) samples and 816 African American (AA) samples (table 1) collected through GenNet, one of the FBPP[8] networks. BP levels, instead of hypertension status, were used as a quantitative trait to increase the power to detect linkage. On the basis of a genomewide linkage scan with the use of microsatellite markers (average density ~10 cM), eight regions had MLOD scores >2: 1q25, 2q33, 10p12, 10p21, 14q13, 14q32, 15q12, and 17q21 (table 3). In six regions, linkage evidence was detected in both EA and AA samples, and the MLODs reported are from the combined data. For 1q25 and 10p12, evidence of linkage was found in EA samples only. We compared our results with those of published genomewide linkage studies for EH and BP-related traits, and we observed that six of the eight linkage regions we identified were confirmed by other studies.[26–32] Additional support for some of these regions was evident from rodent BP-related QTLs identified through crossing inbred hypertensive and normotensive, or salt-sensitive and salt-resistant, rat and mouse strains.[16–18,20,33]

The strongest signal (MLOD 3.2) in our study was the mapping of DBP variation to 182 cM on chromosome 1q in GenNet EA samples. Initial evidence of this linkage in the GenNet study was based on 65% of the samples presently available.[9] Linkage between BP-related phenotypes and the same region on 1q has been reported by three independent studies of the human.[28,30,34] Multiple studies of QTL mapping in mouse and rat all found evidence of BP-related QTLs in the precise homologous region to human 1q.[16–18] Literature review of human and animal BP linkage studies revealed that heterogeneity in study design explains why some but not all studies found the chromosome 1q locus (details available on request).

Fine Mapping of Chromosome 1q Linkage Region

The conventional confidence interval (MLOD-1) of 1q linkage spans 57 cM and 48 Mb in genetic and physical distances, respectively. To refine this genetic interval, we genotyped 16 additional microsatellite markers. The original linkage peak resolved into two distinct peaks, the first with an MLOD of 4.3 at 175.6 cM and the second with an MLOD of 1.8 at 218.5 cM (for unmedicated individuals only) (fig. 1).

Association Study of Nine Positional Candidate Genes

From the 430 RefSeq genes in the 48-Mb region, we focused on 9 genes, chosen either because they are located in close proximity to one of the linkage peaks or because they are located in the homologous minimal linkage region defined by mouse BP-related QTLs: *RGS4, RGS5, ATP1B1, SELP, SELL, SELE, NPHS2, ADORA2,* and *REN* (fig. 1). Fifty-eight SNPs located in or near these nine candidate genes were genotyped in all GenNet AA and EA individuals, to test for association with BP-related phenotypes (SNP-related information detailed in table 4). GenNet probands have high to high-normal BP values but are not necessarily clinically hypertensive. Therefore, we used family-based association tests that use quantitative traits and family information, since these tests have more power than do traditional tests of association. Representative results based on single SNPs and on haplotypes constructed from multiple SNPs are shown in figure 2. When DBP, the phenotype that yielded the most significant linkage result, was analyzed, significant association was detected for one gene, *ATP1B1* (most significant FBAT $P = .002$, for SNP23–SNP24) (fig. 2*A*). Association was detected not only in the linked EA samples but also in the AA samples, despite the lack of linkage evidence. This is not surprising, given the greater power of association tests, compared with that of linkage tests, to uncover common variants; moreover, GenNet AA samples included fewer parents and were thus underpowered to detect linkage.

When SBP was tested as the phenotype, associations with an *ATP1B1* SNP became more significant ($P = .0006$, for SNP26 in EA samples) (fig. 2*B*), despite the fact that there was less evidence of linkage to SBP (MLOD 1.3). In addition, we found significant associations with SBP for two other genes in the AA samples. In *RGS5,* SNP3–SNP11 are associated with SBP in AA samples (the most significant

Table 4. Information on 58 Candidate-Gene SNPs

The table is available in its entirety in the online edition of *The American Journal of Human Genetics.*

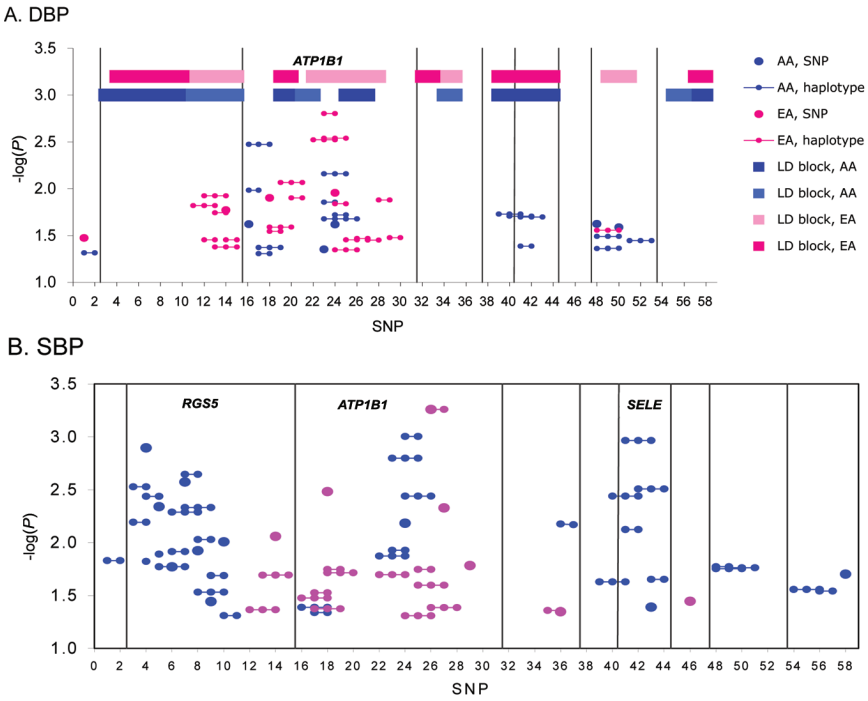

Figure 2. Association study of 58 candidate-gene SNPs with DBP (*A*) and SBP (*B*), on the basis of unmedicated BP values. Only *P* values <.05 are plotted as $-\log_{10}(P)$. *P* values derived from GenNet EA samples (*pink circles*) and AA samples (*blue circles*) are shown separately. *P* values from haplotype-based analyses are denoted as circles connected by lines. SNPs in each of the nine genes—*RGS4, RGS5, ATP1B1, SELP, SELL, SELE, NPHS2, ADORA1,* and *REN*—are demarcated on the *X*-axis. The horizontal bars indicate SNPs in LD blocks estimated using 377 unrelated EA samples (*pink and red*) and 276 AA samples (*blue*).

$P = .001$, for SNP4), and SNP12–SNP15 are associated in EA samples (most significant $P = .009$, for SNP14). Variants in *SELE* also demonstrated significant association for AA samples, more so when haplotypes of multiple SNPs were analyzed (for SNP43, $P = .04$; for haplotype of SNP41–SNP43, $P = .001$). Lack of consistent linkage and association signals in GenNet AA and EA samples suggests some degree of heterogeneity between these two samples.

RGS5 spans across 60.8 kb, and the 13 SNPs genotyped are in two LD blocks (figs. 2 and 3). SNP3–SNP10, in the first LD block, are associated with BP in GenNet AA samples, whereas SNP11–SNP15, in the second LD block, show significant association with BP in the EA samples (fig. 2). In *ATP1B1,* SNP18 and SNP26 are both associated with BP, yet there is little evidence of LD between these two SNPs (fig. 3). Importantly, haplotype-based association of these two SNPs with SBP was more significant than that of the single SNPs (in EA samples, $P = .009$, .0006, and .0002, for SNP18, SNP26, and the haplotype, respectively). Although the resolution is limited by the LD between SNPs, on the basis of these patterns of association, it is likely that there are multiple functional sites in *ATP1B1* and *RGS5* that influence BP.

The 58 SNPs that we genotyped are from functionally relevant hypertension candidate genes located in a replicated linkage region. Nevertheless, to avoid type I errors from multiple comparisons, we corrected the *P* values of the association signals for these three genes, to account for testing of multiple SNPs, many of which are in strong LD with each other. After correction, the strongest association signals (smallest *P* values) for all three genes remained significant: SNP4 (*RGS5*) in AA samples, SNP26 (*ATP1B1*) in EA samples, and haplotype of SNP41–SNP43 (*SELE*) in AA samples.

To further validate the association signal in these three genes, we replicated our findings in independently collected samples from two other FBPP Networks, GENOA and HyperGEN (the total number of samples studied was 4,079) (table 1). Nine SNPs in *ATP1B1, RGS5,* and *SELE,* associated either individually or as haplotypes with $P <$ .01, were genotyped and analyzed using FBAT. We detected significant association with BP in all three genes in at least one sample group (table 5). Overall, we detected evidence of association for *RGS5* in four sample groups (GenNet EA and AA samples and HyperGEN EA and AA samples), with the strongest signal from SNP7 ($P = .002$

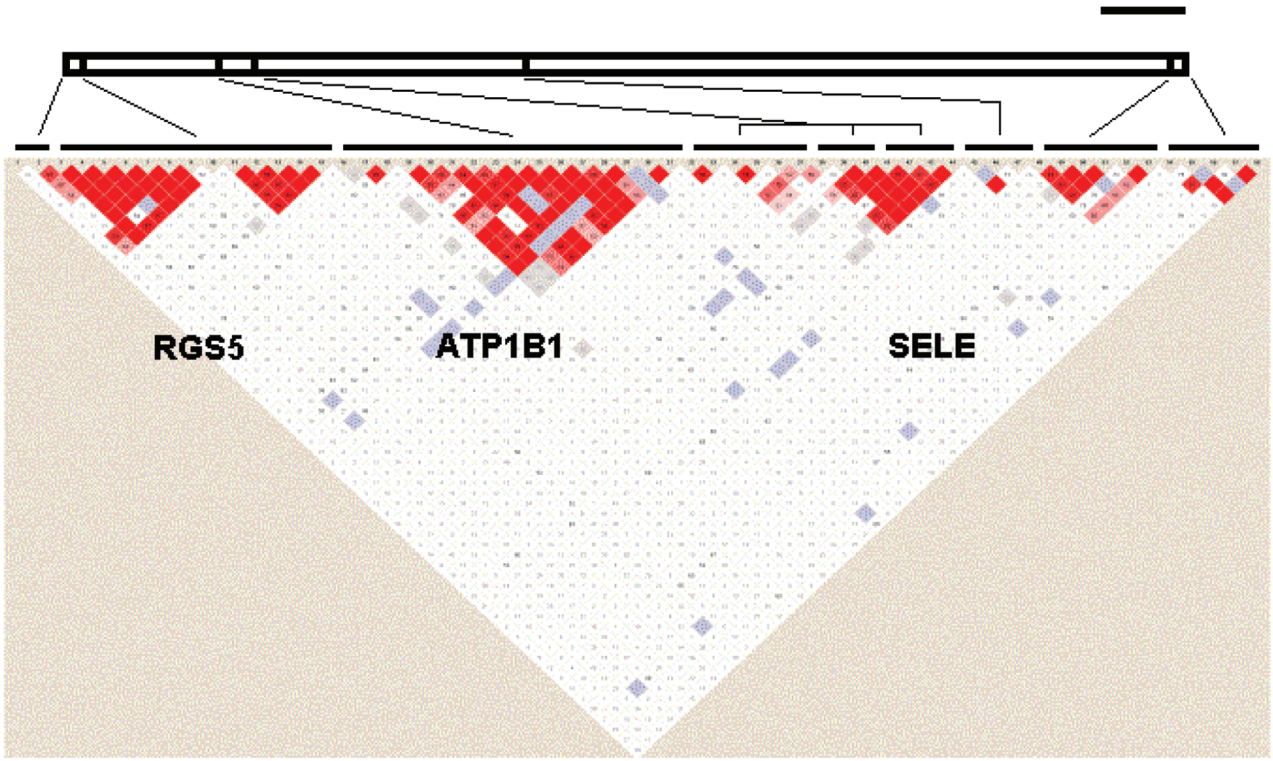

Figure 3. Variants in *RGS5, ATP1B1,* and *SELE* independently associated with BP. Vertical lines in the horizontal bar denote the physical relationships of candidate genes tested. The black line at top right indicates 2 Mb. *RGS5* and *ATP1B1* are separated by ~5.96 Mb, and *ATP1B1* and *SELE* by 616 kb. The pairwise LD, as measured by D', was calculated using Haploview (on the basis of unrelated GenNet EA samples), and D' is shown as $100 \times D'$. Cells are blank where $D' = 1$, bright red indicates high LD ($D' = 1$; LOD ≥ 2), pink indicates weaker LD ($D' < 1$; LOD ≥ 2), white indicates no LD ($D' < 1$; LOD < 2), and blue indicates noninformative results ($D' = 1$; LOD < 2). Similar lack of LD between these three genes is observed in GenNet AA samples and HapMap samples (data not shown).

Table 5. Associations between *RGS5, ATP1B1,* and *SELE* SNPs and BP Levels in the Original (GenNet) and Replication (HyperGEN and GENOA) Samples

Gene and SNP	GenNet				HyperGEN				GENOA			
	EA		AA		EA		AA		EA		AA	
	SBP	DBP	SBP	DBP	SBP	DBP	SBP	DBP	SBP	DBP	SBP	DBP
RGS5:												
4	…	…	.001[b]	…	…	…	…	…	…	…	…	…
5[a]	…	…	.005	…	…	…	.04	.02	.07	…	…	…
6	…	…	.02	…	…	…	…	…	…	…	…	…
7[a]	…	…	.002	.08	.001[b]	.001[b]	.02	.05	…	…	…	…
8	…	…	.01	…	…	…	…	…	…	…	…	…
9	…	.08	.04	…	…	…	…	…	…	…	…	…
10[a]	…	…	.01	…	…	…	.04	.03	.07	…	…	…
14	.009	.02	…	…	…	…	…	…	…	…	…	…
ATP1B1:												
16	…	…	…	.02	…	…	…	…	…	…	…	…
17	…	…	…	.06	…	…	…	…	…	…	…	…
18[a]	.003	.01	…	…	.06	.05	…	…	…	.02	…	…
19	…	.06	…	.09	…	…	…	…	…	…	…	…
20	…	.06	…	…	…	…	…	…	…	…	…	…
21	…	.05	…	…	…	…	…	…	…	…	…	…
23	…	…	.06	.04	…	…	…	…	…	…	…	…
24[a]	…	.01	.007	.02	…	…	…	…	…	…	…	.03
26[a]	.0006[b]	.03	…	…	…	…	…	…	…	…	…	…
27[a]	.004	.03	…	…	…	…	…	…	…	…	…	…
28	…	.03	…	…	…	…	…	…	…	…	…	…
29	.02	.008	…	…	…	…	…	…	…	…	…	…
SELE:												
42[a]	…	…	.07	…	…	…	…	…	…	…	.01[b]	.002[b]
43[a]	…	…	.04	…	…	…	…	…	…	…	…	…

NOTE—Only SNPs with at least one FBAT *P* value < .10 are shown. Cells with an ellipsis (…) are those with FBAT *P* values < .10 and those not genotyped in the replication samples. SBP and DBP were age and sex adjusted. Medicated individuals were removed before analysis of GenNet samples, and their data were adjusted on the basis of average treatment effect for analysis of HyperGEN and GENOA samples.

[a] SNPs genotyped in the replication samples.

[b] Associations that remain significant ($P < .05$) after Bonferroni correction for the number of independent SNPs tested.

and .001, for GenNet AA samples and HyperGEN EA samples, respectively). For *ATP1B1,* significant associations were detected in four sample groups (GenNet EA and AA samples and GENOA EA and AA samples), with the strongest signal from SNP26 ($P = .0006$, for GenNet EA samples). For *SELE,* we detected association in two sample groups, GenNet AA and GENOA AA samples, with the strongest signal from SNP42 ($P = .002$, for GENOA AA samples). In these replication samples, association between SNP markers and SBP and DBP was significant for both phenotypes (lower *P* values were observed for DBP; data not shown). Importantly, for all SNPs that were significantly associated with BP levels in multiple samples, the associations were in the same direction (for example, for SNP24 in GenNet AA and EA samples [fig. 2*A*] and SNP42 in GenNet EA and GENOA AA samples [table 6]). Replicated association signals are population specific; what was significant for GenNet AA samples tended to be significant for the AA samples of the replication samples (for SNP5, SNP7, SNP10, SNP18, SNP24, and SNP42) (table 5). Furthermore, the association signals seen in *RGS5* and *SELE* (SNP7 and SNP42) were significant even after correction for multiple testing.

Phenotypic Effect Sizes of Hypertension-Susceptibility Alleles

Locus-specific phenotypic effects estimated from the same population in which linkage was originally found are sometimes inflated.[35] Therefore, we estimated the phenotypic effect of *RGS5, ATP1B1,* and *SELE,* using not only samples from GenNet but also samples from GENOA and HyperGEN. We found significant and quantitatively similar effect sizes for all three genes in the GenNet and GENOA samples, with age- and sex-adjusted SBP effects ranging from 1.5 to 5.1 mm Hg at individual SNPs (table 6). The HyperGEN samples, because of their smaller size, have relatively less power to estimate the phenotypic effect size. Nevertheless, analysis of GENOA and HyperGEN samples together yielded the same effect sizes for SNP5, SNP10, and SNP42 as did analysis of GENOA samples alone (data not shown). Furthermore, whenever the phenotypic effects of multiple genes were significant in the same populations, we observed incremental increases in mean BP in those samples, with more alleles associated with higher BP (susceptibility alleles). For example, when GenNet EA samples were grouped by their *ATP1B1* and *SELE* genotypes, their mean age- and sex-adjusted SBP in-

Table 6. Phenotypic Effect Sizes of SNPs Associated with BP

Gene and SNP	Effect (mm Hg)	*P*	Samples
RGS5:			
5	1.7	.04	GENOA AA
6	1.8	.09	GenNet AA
7	2.2	.03	GenNet AA
8	2.1	.04	GenNet AA
9	3.6	.06	GenNet AA
10	1.5	.08	GENOA AA
ATP1B1:			
23	4.3	.0005	GenNet EA
23	5.1	.04	GenNet AA
26	3.9	.001	GenNet EA
27	3.5	.005	GenNet EA
SELE:			
42	2.7	.04	GenNet EA
42	2.3	.006	GENOA AA
43	1.8	.008	GenNet EA
44	1.7	.04	GenNet EA

NOTE.—The estimated phenotypic effects are age- and sex-adjusted SBP in mm Hg. Effect sizes with *P* values <.10 are listed. For GenNet samples, only SBP values of unmedicated individuals were used for analysis. For GENOA samples, SBP values adjusted for medication effect were used in the analysis.

creased from −3.2 to 7.0 mm Hg with each additional susceptibility allele. This observation indicates that the effects of multiple genes on BP are cumulative (fig. 4). The combined effects on SBP are 10.2 and 7.8 mm Hg for *ATP1B1*+*SELE* and *RGS5*+*SELE,* respectively.

In analyzing multiple study populations, some of which were ascertained for BP values and some for hypertension status, we encountered the problem of estimating true BP levels from medicated individuals. The effect of treatment was studied by using hypertension status instead of BP values, by removing samples whose BP values are altered by medication, by including them with the use of treated BP values, or by including them with the use of BP values adjusted by an estimated average treatment effect.[12] We found that, although *P* values varied, the overall pattern of association remained the same (data available on request). Because the percentage of medicated subjects in GenNet is relatively small (22%), removal of medicated individuals from the analysis resulted in the most significant *P* values (fig. 2 and table 5). In contrast, 63% and 36% of GENOA and HyperGEN subjects, respectively, were taking hypertension medication. In this situation, exclusion of medicated individuals led to significant reductions in power, and the most significant associations were detected using BP values adjusted for average medication effect.

Evidence That the Three Genes Are Independently Associated with BP

The three associated genes are physically distant from each other (*RGS5*–5.96 Mb–*ATP1B1*–616 kb–*SELE*). Importantly, there is no evidence of LD or association between SNPS in these three genes. (Pairwise *D*′ values are shown in fig. 3. All pairwise values of r^2 for SNPs located in different genes were <0.02.) In addition, conditional association tests that analyzed only samples with the same genotype in one gene (for example, SNP4 of *RGS5* in GenNet AA samples) still demonstrated significant association in the other two genes (data not shown).

Discussion

This study establishes that there are multiple BP-determining genes in the 1q linkage region. Importantly, it is likely that most susceptibility alleles of complex diseases have, individually, modest effects similar to those of the *RGS5, ATP1B1,* and *SELE* alleles we studied. Hence, the linkage signals uncovered by most genomewide linkage studies likely arise from multiple genes in the same region that have a combined phenotypic effect detectable by the study sample size. In fact, the combined phenotypic effects of *RGS5, ATP1B1,* and *SELE* account for only a fraction of the BP variants attributed to the 1q linkage region (data not shown), and there are other, unidentified BP-regulating genes in 1q. We hypothesize that clustering of multiple genes that affect the same phenotype might be, in some cases, evolutionarily advantageous. If so, such regions can be identified by comparison of linkage signals from multiple species, as we have done for BP-related loci from human, mouse, and rat hypertension studies.

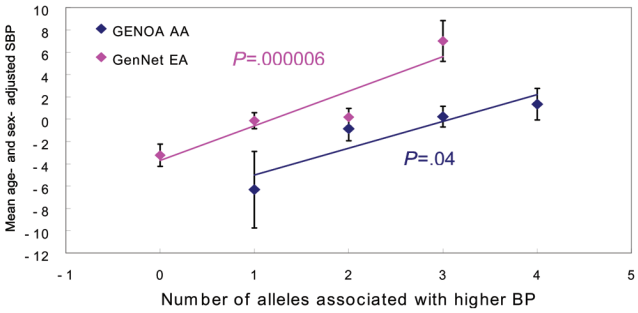

Figure 4. Cumulative phenotypic effects of *ATP1B1, RGS5,* and *SELE* alleles. The effects of *ATP1B1* and *SELE* alleles are both significant in GenNet EA samples (for SNP26 and SNP43). The mean adjusted SBP in those with zero, one, two, and three alleles associated with higher BP increased from −3.2 to 7.0 mm Hg incrementally with every additional susceptibility allele. The numbers of GenNet EA individuals with zero, one, two, and three copies of susceptibility alleles are 95, 352, 275, and 41, respectively. For *RGS5* and *SELE* alleles in GENOA AA samples (for SNP5 and SNP42), the mean SBP increased from −6.3 to 1.3 mm Hg incrementally with every additional susceptibility allele. The number of GENOA AA individuals with one, two, three, and four copies of susceptibility alleles are 52, 470, 673, and 266, respectively. In both cases, the best-fitting line and the *P* values from Student's *t* test between those with the fewest and those with the most susceptibility alleles are also shown.

The combined phenotypic effect of these 1q genes, estimated to be 8–10 mm Hg for SBP, is comparable to some rare monogenic forms of hyper- and hypotension.[36,37] For example, the mitochondrial tRNA mutation that causes hypomagnesemia, hypertension, and hypercholesterolemia (MIM 500005) has been found in a single family and is associated with an age- and sex-adjusted SBP difference of 13 mm Hg.[36,37] In contrast, hypertension-susceptibility alleles in *RGS5, ATP1B1,* and *SELE* are relatively common (minor-allele frequencies range from 0.08 to 0.51) (table 4). Moreover, the combined phenotypic effect is similar to several mouse models of hypertension, in which genes central to BP regulation, such as *AGT* (MIM 106150),[38] *NPR1* (MIM 108960),[39] *NPPA* (MIM 108780),[40] *NPR2* (MIM 108961),[41] and *AGTR1* (MIM 106165)[42,43] have been deleted or duplicated. In these models, average BP difference per copy ranges from 5 to 12 mm Hg. Furthermore, the magnitude of the observed effect is similar to the average effect of current hypertension treatment regimens on SBP (a single medication at standard dosage).[44] Consequently, by physiological criteria, we have identified a locus with major effects and common variants.

How are these three genes related to BP regulation? *ATP1B1* encodes the ubiquitously expressed β subunit of Na,K-ATPase, an intrinsic oligomeric protein necessary for the maintenance of Na^+ and K^+ electrochemical gradients across the plasma membrane. This transporter is involved in multiple BP-regulating physiological processes: renal sodium reabsorption, vascular smooth-muscle-tone regulation, and cardiac muscle contraction. Decreased Na,K-ATPase activity precedes the development of hypertension in animal models.[45] Although the α subunit has the catalytic activity of the enzyme, the β subunit is required for the proper cellular location of the enzyme and its stability. In many cells and tissues, β mRNA is less abundant than α mRNA, suggesting that the translation of β mRNA is a crucial regulatory step in determining the abundance and activity of this important enzyme.[46–49]

Regulators of G-protein signaling (RGSs) are a family of proteins that promote GTPase activity of G-protein–coupled receptors. *RGS5* has several potential roles in BP regulation. First, RGS5 inhibits G-protein signaling by inactivating Gα(q) and Gα(i), which mediate vasoconstrictors such as angiotensin II and endothelin-1.[50,51] Second, *RGS5* is down-regulated during morphogenesis of the developing vasculature, and its expression is elevated in tumor-associated blood vessels, suggesting a role in angiogenesis.[52,53] Third, *RGS5* might play a role in the sensing of hemodynamic change and the remodeling of arteries.[54] In addition, *RGS5* is down-regulated in stroke-prone spontaneously hypertensive rats, compared with in stroke-resistant hypertensive rats, and is implicated in the pathogenesis of stroke.[55]

SELE encodes E-selectin, an endothelium-specific adhesion molecule and a marker of endothelial function. Endothelial dysfunction, or impaired endothelium-dependent vasodilation, and elevated plasma levels of soluble E-selectin are both common features of EH.[56,57] Activated endothelium promotes the formation of atheroma, reduces the elastic properties of the arterial wall, and alters responsiveness to vasoactive stimuli. Endothelial dysfunction is likely a primary event in the development of EH, and endothelial damage is exacerbated by the shear stress of chronically elevated BP.[58,59]

Instead of a purely positional-cloning approach, we opted to prioritize our association study by focusing on genes with known or putative functions that are compatible with known BP-regulation physiology. The associations between these three genes and BP levels have been replicated in other samples. Analysis of all genes in the 1q linkage region can only confirm and will not diminish our findings.

In *ATP1B1,* strong LD extends from SNP19 to SNP31, a 57.4-kb region that starts at intron 2 of *ATP1B1* and ends at the last two exons of an overlapping gene, *NME7*. Although the SNPs located in *ATP1B1* are more significantly associated with BP levels than are SNPs in *NME7,* we have not excluded *NME7* from playing a role in BP regulation. The same is true for the association observed between *SELE* SNPs and BP; the associated *SELE* SNPs are in strong LD with SNPs in *SELL,* a related gene that encodes lymphocyte adhesion molecule 1, L-selectin.

We have not established which SNP in these three genes are functionally responsible for BP-level differences. With the exception of SNP42, a nonsynonymous coding SNP in *SELE,* other SNPs significantly associated with BP levels are located in either introns or UTRs. Some of these SNPs are located in regions that are evolutionarily conserved. For example, the most significantly associated SNP in our study, SNP26, is located in the 3′ UTR of *ATP1B1,* a region where human and distantly related species, such as torpedo fish, share a high degree of sequence identity.[60] We suspect that the variants at this site or another site in LD with SNP26 affect mRNA stability, translation efficiency, or some other 3′-UTR–related function. Experiments to confirm this hypothesis and to identify functional variants in *RGS5* and *SELE* are underway.

So far, the identified genes underlying the rare and monogenic forms of hypertension and hypotension are all genes involved in renal sodium reabsorption. However, the genes responsible for BP regulation and EH susceptibility are not similarly limited in their functions, because physiological studies have long established the importance of the vascular system in BP control and because endothelial dysfunction and capillary rarefaction are both hallmarks of EH.[56,61] The findings of this study establish a genetic association between BP regulation and genes involved in vascular functions. Indeed, preliminary studies indicated that genotypes of *RGS5, ATP1B1,* and *SELE* are associated with varying levels of vascular responses to stress (data not shown). Ultimately, knowledge of both the genes that influence cardiac output through renal salt-water balance and the genes that determine systemic re-

sistance by modifying peripheral vasculature is important for an understanding of EH.

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Web Resources

The URLs for data presented herein are as follows:

Comparative Maps, http://www.ncbi.nlm.nih.gov/Homology/ (for human-mouse homology map)
dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/
deCODE, http://www.decode.com/ (for genotyping service)
FBPP, http://www.biostat.wustl.edu/fbpp/FBPP.shtml
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for EH, *RGS4, RGS5, ATP1B1, SELL, SELE, SELP, REN, ADORA1, NPHS2,* hypercholesterolemia, *AGT, NPR1, NPPA, NPR2,* and *AGTR1*)
Primer3, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi/
UCSC Genome Browser, http://genome.ucsc.edu/cgi-bin/hgGateway

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