Erythrocyte Sodium-Lithium Countertransport and Blood Pressure
A Genome-Wide Linkage Study

Alan B. Weder, Maria Carolina Delgado, Xiaofeng Zhu, Lillian Gleiberman, Donghui Kan, Aravinda Chakravarti

Abstract—Increased activity of erythrocyte sodium-lithium countertransport is associated with essential hypertension. Sodium-lithium countertransport is highly heritable, but no single gene product mediating the exchange or explaining the association of increased sodium-lithium countertransport activity and hypertension has been identified. We performed a linkage study by using erythrocyte sodium-lithium countertransport as a quantitative phenotype and genome-wide markers at an average resolution of \(\approx 10\) cM to identify quantitative trait loci explaining sodium-lithium countertransport activity. A peak LOD score of 2.83 was detected on chromosome 15q at D15S642, a marker previously shown to be linked to blood pressure. Several genes mapped to this region are possible candidates for factors affecting erythrocyte sodium-lithium countertransport and/or blood pressure. Further studies confirming the presence of a quantitative trait locus in this region and evaluating these candidate genes may help explain the association of elevated sodium-lithium countertransport and hypertension. (Hypertension. 2003;41[part 2]:842-846.)

Key Words: hypertension, essential | erythrocytes | membranes | genes | genetics

In 1980, Canessa et al\(^1\) reported an association between elevated activity of erythrocyte sodium-lithium countertransport (SLC) assayed under carefully defined conditions and essential hypertension, an observation that has been confirmed repeatedly. SLC is continuously distributed with right-skewing in populations, and biometric mixture analysis has suggested that the skewed distribution of SLC is composed of 2 overlapping normally distributed subpopulations.\(^2-5\) In addition to blood pressure, a number of exogenous factors and traits are associated with variation in SLC activity, including plasma triglyceride\(^6\) and potassium\(^7\) levels, insulin resistance,\(^8\) ethnicity,\(^9\) pregnancy,\(^10\) and nonmodulation of blood pressure in response to changes in dietary sodium.\(^11\) Numerous other potential confounders have been examined, often with inconsistent findings,\(^12\) but it is safe to say that at present, no single trait clearly explains the finding of 2 subpopulations.

Most genetic analyses support a high heritability (\(h^2\)) for SLC, reportedly in the range of 60% to 80%\(^13-15\) (although in some populations \(h^2\) is much lower after adjustment for covariates\(^16\)). Complex segregation analyses have provided some evidence for a major gene effect,\(^2,13\) but not all studies agree.\(^14\) In both population\(^2,16\) and family studies,\(^13\) elevated SLC has been suggested to be a heterogeneous phenotype.\(^15\) The unknown transmembrane protein mediating SLC may interact with \(\alpha\)-adducin; the combination of high SLC and the \(\alpha\)-adducin 460Trp allele defines a subpopulation phenotypically distinct from that with high SLC and the Gly-Gly genotype.\(^17\) Tropomysin, which may interact with both the transporter mediating SLC and \(\alpha\)-adducin, has been identified as a possible mediator of increased SLC.\(^18\)

Because the ionic requirements and mode of transport (one-for-one transmembrane exchange) are similar to those of sodium-proton antipporter (NHE), the suggestion was forwarded that SLC is a mode of NHE,\(^19,20\) and a sub-pair linkage analysis of the ubiquitous NHE-1 exchanger was undertaken as one of the first studies of a candidate gene for hypertension outside the renin-angiotensin system.\(^21\) This linkage analysis excluded NHE-1 as a cause of increased SLC or hypertension, and the negative findings have been confirmed.\(^22\) Linkage with the MN blood group antigen on chromosome 4 in men but not women was reported in a small sub-pair analysis,\(^23\) but no association with MN haplotypes was observed in another study.\(^24\) Kammerer et al\(^25\) recently reported significant linkage of SLC and a site on chromosome 5 in baboons that is homologous with a region of chromosome 4 in humans. SLC activity does not appear to differ by ACE genotypes,\(^26\) but an association of elevated SLC with the Trp64Arg polymorphism within the \(\beta\)-3 adrenergic gene has recently been observed.\(^27\)
The present report is from the GenNet study, which is part of the NHLBI-sponsored Family Blood Pressure Program. The goal of this Program is to discover genes for essential hypertension (see http://www.hypertensiongenetics.org). One of the intermediate phenotypes assessed in the GenNet Tecumseh center is erythrocyte SLC. We report here genome-wide scan results by a variance component approach, using SLC as a quantitative trait.

Methods

Population

A description of the population studied is to be published (Thiel BA, Chakravarti A, Cooper RS, Lake A, Lewis S, Lynn A, Tiwari H, Schork NJ, Weder AB, unpublished data, 2002). Briefly, participants are white residents of Tecumseh, Mich. Records of families involved in previous studies in Tecumseh were used to identify male and female subjects between 25 and 40 years old. Within this group, proband eligibility was defined as having a recorded systolic or diastolic blood pressure in the upper 15% of the blood pressure distribution, using gender-specific criteria. Because we used blood pressure as a quantitative phenotype in the GenNet study, treatment with antihypertensive medications excluded subjects from this analysis, as did the finding of an elevated serum creatinine (>1.5 mg/dL), diabetes mellitus, pregnancy, or serious medical illness. Siblings and parents of probands were recruited without regard to blood pressure status. All subjects gave written informed consent as approved by the University of Michigan Institutional Review Board before participation in the study. Our final data include 127 nuclear families, with a total of 163 sib-pairs.

Blood Pressure and Other Physical Measures

Blood pressure measurements were made with a standardized protocol by a single observer previously trained and certified in blood pressure measurement technique. Two manual measurements were obtained for each subject with a standard mercury manometer, and the average systolic and diastolic blood pressures were used in this study. Height was measured by using a wall-mounted tape measure, and weight was determined on a balance-beam scale. Body fat was determined by bioelectrical impedance and body fat distribution by the waist-hip ratio.

Erythrocyte Sodium-Lithium Countertransport

SLC was measured as the difference in lithium efflux rate from lithium-loaded cells into sodium chloride and sodium-free media, as described in an earlier publication.9 The only change in methodology was a switch from a magnesium chloride-based, sodium-free medium to a 150-mmol/L choline chloride-based solution for the estimation of sodium-independent lithium efflux. Concern has been raised that use of the traditional magnesium chloride medium may result in suppression of sodium (lithium)-potassium-chloride co-transport and thereby overestimate SLC.26 We calculated 2 measures of SLC, the traditional measure of the difference of lithium efflux rates into 150 mmol/L sodium chloride and choline chloride (SLC-1) and the maximal empirically observed rate into several different concentrations (37.5, 75, 112.5, 150 mmol/L) of sodium chloride (SLC-2). The 2 measures are highly correlated (r=0.99, P<0.0001), and use of the highest observed value decreases the random error associated with any one rate determination near the maximal activity of the transporter.

Genotyping

A set of 387 short-tandem repeat markers (Set 9, www.marshmed.org/genetics) was genotyped by the Mammalian Genotyping Service (MGS) in Marshfield, Wis.29 The mean heterozygosity for these markers is ~76%, with an average sex-equal distance of 10 cM on the genetic map.29 DNA samples extracted from citrated buffy coats were provided to the MGS in 3 batches, and extensive quality checks were carried out to verify consistency of marker genotyping and stated pedigree relationships. First, PEDCHECK20 was used to check for mendelian inheritance. ASPEX/KINSHIP33 was then used to estimate the biological relationship maximum likelihood (ML) among individuals in the pedigrees based on all marker data. The relationships were reassigned if they were clearly misclassified. Errors identified in PEDCHECK not based on misclassification of pedigrees were assumed to have occurred in the genotyping process, and the associated markers were set to missing among the appropriate family members.

Statistical Analysis

The technique of mixture analysis has been described (http://anymouse.uchicago.edu/pailesource/code.php?stage=download&project_no=12). This program analyzes distributions that are hypothesized to be a mixture of subdistributions. The goals in analyzing finite mixture models are 2-fold: (1) to determine what model best fits the data at hand (eg, a mixture of 1, 2, or 3 normal distributions) and (2) to estimate the parameters of that best-fitting model. In practice, these steps are performed in reverse order: parameters are first estimated, and the solutions for different models are then compared. This program uses an ML approach to estimate the parameters of the each sub-distribution. The hill-climbing routine that is used to find the ML estimates is an expectation-maximization (EM) algorithm. Any 2 solutions to the same model (eg, a 2-group model) can be compared directly through their likelihood (or the natural logarithm of the likelihood, the log-likelihood). This program follows McLachlan and Basford33 in using parametric bootstrapping to compare solutions to different models. Note that the χ² approximation and Akaïke’s information criterion are not valid for comparing models with different numbers of groups.

This analysis (see Results) demonstrated that 2 overlapping normal distributions best explained the overall population distribution, and subjects were subsequently classified as having “high” or “low” countertransport. Group differences were assessed with the Student t test, adjusting for age and gender; significance was accepted at P<0.05.

Genetic Linkage Analysis

Genome-wide linkage analysis of SLC was performed by using the multipoint variance component program in GENEHUNTER2.34 The variance component method in GENEHUNTER2 specifies the expected genetic covariances between relatives as a function of their identity-by-descent (IBD) relationships at a marker locus. The IBD probabilities were estimated from all available genotyped marker loci. The likelihood ratio test was applied to test the null hypothesis of no additive genetic variance due to a quantitative trait locus (QTL). Sex, age, age² and body mass index (BMI) were incorporated as covariates, and their effects were simultaneously estimated by the ML method. In all analyses, allele frequencies were estimated from the marker data. We used MGS map distances in the linkage analysis. For the region with linkage evidence found in the multiple-point analysis, we also conducted single-marker analysis with the use of the program SIBPAL2 in S.A.G.E. (S.A.G.E. [2002] Statistical Analysis for Genetic Epidemiology, 4.0. Computer program package available from the Department of Epidemiology and Biostatistics, Rammelkamp Center for Education and Research, Case Western Reserve University, Cleveland, Ohio).

To assess the empirical significance of our results, we performed a simulation study. Retaining the pedigree and phenotype data, we simulated marker genotype data based on the observed marker allele frequencies. One hundred replications were generated and analyzed by GENEHUNTER2.

Results

Characteristics of the study population and the 2 subpopulations derived from mixture analysis are shown in Table 1. The analysis supporting the presence of 2 as opposed to 1 or 3 subpopulations is shown in Table 2. These population characteristics are similar to those we have observed in the
Linkage analysis was performed with SLC used as a quantitative phenotype. The additive heritabilities of SLC-1 and SLC-2 were 0.42 and 0.49, respectively. Figure 1 shows the genome wide LOD scores by variance component analysis for SLC-2 when gender, age, age2, and BMI were adjusted. The largest LOD score, 2.83 (unadjusted point-wise probability value, 0.0002), was found at marker D15S642. Simulated 100 replicate data sets. The probability value (genome-wide) is 0.1 for an LOD score of 2.83.

**Discussion**

Although we did not find a chromosome region with a 5% genome-wide significance level according to the criteria of Lander and Kruglyak,36 our analysis of SLC did reveal suggestive linkage evidence (LOD = 2.83) on chromosome 15 (15q26). Interestingly, in an extreme low concordant sib-pair analysis with blood pressure, Xu et al37 found a LOD score of 2.69 in this region in a Chinese population.

By refining the trait definition and genotyping additional markers, Xu et al38 further detected significant linkage evidence with a LOD score of 3.77 at 9 cM away from D15S642 in the same region. Our analysis yielded a maximum LOD score at marker D15S642. Simulation of 100 replicates under a null hypothesis of no linkage evidence in this region yielded maximum LOD scores all <2.83, indicating our finding of a LOD score of 2.83 corresponds to a significant empirical probability value of <0.01 for a replication study.36 When BMI was not included as a covariate, the LOD score was 2.7, indicating that the linkage evidence is not due to the correlation between SLC and BMI. Together with previous linkage evidence for blood pressure provided by Xu et al,37,38 our analytic results suggest that a QTL in this

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**TABLE 1. Characteristics of All Subjects and Classified by Low and High SLC**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All Subjects (n=357)</th>
<th>Low SLC (n=192)</th>
<th>High SLC (n=165)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>44±1 (13–84)</td>
<td>44±1</td>
<td>45±1</td>
<td>NS</td>
</tr>
<tr>
<td>Male:female</td>
<td>159:198</td>
<td>83.76</td>
<td>109.89</td>
<td>NS</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>76.4±0.5 (48–108)</td>
<td>74.5±0.7</td>
<td>78.5±0.7</td>
<td>0.0001</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>121.6±0.7 (88–199)</td>
<td>119.4±1.1</td>
<td>124.2±1.1</td>
<td>0.002</td>
</tr>
<tr>
<td>Height, cm</td>
<td>169.3±0.5 (147.2–197.8)</td>
<td>168.6±0.5</td>
<td>170.0±0.5</td>
<td>0.03</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>84.0±1.1 (41.8–187.7)</td>
<td>79.0±1.3</td>
<td>89.7±1.4</td>
<td>0.0001</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>29.2±0.3 (16.5–58.5)</td>
<td>27.7±0.4</td>
<td>30.9±0.5</td>
<td>0.0001</td>
</tr>
<tr>
<td>Waist:hip</td>
<td>0.854±0.005 (0.630–1.400)</td>
<td>0.844±0.005</td>
<td>0.866±0.005</td>
<td>0.003</td>
</tr>
<tr>
<td>Fat mass, kg</td>
<td>30.0±0.7 (6.1–108.0)</td>
<td>27.2±0.9</td>
<td>33.3±1.0</td>
<td>0.0001</td>
</tr>
<tr>
<td>SLC</td>
<td>0.248±0.006 (0.007–1.044)</td>
<td>0.170±0.006</td>
<td>0.340±0.006</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Values are mean±SE, adjusted for age and gender; P for High vs Low; NS = not significant. DBP indicates diastolic blood pressure; SBP, systolic blood pressure; BMI, body mass index; SLC, sodium-lithium countertransport, mmol/L cell/h.

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**TABLE 2. Results of the Maximum Likelihood Analysis for the One- Through Three-Group Models for the SLC Data**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Bootstrap Test (H₁ vs H₂)</th>
<th>Observed −2Δ</th>
<th>Bootstrap Results</th>
<th>Bootstrap P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td></td>
<td>94.04</td>
<td>0/300</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>0.0152</td>
<td>300/300</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*Bootstrap Results* indicate the number of bootstrap replicates that exceeded the observed value for the log-likelihood ratio (−2Δ), out of the total number of replicates performed (300).

*Model with fewer groups (H₁) is rejected in favor of a model with more groups (H₂), at the α=0.05 level.

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**Figure 1. LOD scores from a genome wide scan of sodium-lithium countertransport using GENEHUNTER2. The numbers under the horizontal axis represent chromosome number with the width being proportional to chromosomal genetic length.**
region may influence both blood pressure and SLC or that one phenotypic measure may affect the other. Since the information content between D15S657 and D15S642 is relatively low (≈0.55) as the result of the relatively large distance between the 2 markers (17 cM), we may have lost some statistical power. However, we are still able to replicate the linkage evidence with a relatively small sample size, suggesting that a gene in this region may play a significant role in regulating blood pressure. Further studies in different populations are necessary to confirm that there is a QTL associated with blood pressure and SLC in this region.

A search of the Genome Database (http://www.gdb.org/gdb) revealed several genes in this region that could be important regarding SLC activity and its relation to hypertension, obesity, and insulin resistance. These include IGFIR, the insulin-like growth factor 1 receptor, IDDM3, which increases susceptibility to insulin-dependent diabetes mellitus and PLIN, the human homolog of rat perilipin, a unique protein associated with intracellular neutral lipid droplets in adipocytes and steroidogenic cells.

Hypertension is a complex disease with multiple determinants, both genetic and environmental, and there is evidence suggesting that each of the polygenes contributing to hypertension has only a modest effect. Whether such genes can be detected by traditional linkage approaches is questionable, since even the Family Blood Pressure Program, which has characterized some 11,357 individuals, has not been able to detect significant linkage between a standardized set of markers and the blood pressure trait (Province MA, Kardia SLR, Ranade K, et al. An interim meta-analysis of genome-wide linkage scans for hypertension: the NHLBI Family Blood Pressure Program [FBPP], unpublished data, 2002). Blood pressure is a less than ideal phenotype for such analyses because it is almost certainly the product of diverse physiological and biochemical mediators and is thus genetically heterogeneous. To improve phenotypic specificity and increase the power for detecting linkage, we focused on SLC, an intermediate phenotype previously associated with hypertension and one that may result from fewer genes and environmental factors, thereby increasing genetic homogeneity. Although we studied only 357 individuals for whom SLC measurements were available, we appear to have substantial power to detect a QTL associated with SLC. Our study confirms the utility of a strategy of defining an intermediate phenotype in linkage analysis for dissecting complex diseases such as hypertension.

The current analysis did not demonstrate linkage with any of the human chromosome 4 markers studied and thus provides no support for the recent report of linkage between SLC and markers on chromosome 5 in baboons. Since species differences in SLC activity have been previously demonstrated, it is possible that SLC is mediated by a different transporter in baboons and humans. Alternatively, it is plausible that the different sites identified in baboons and humans may represent different factors involved in SLC regulation. We also acknowledge that our relatively small sample size may not have been adequately powered to detect a QTL in this region. Finally, we have recently reported an apparently different relation of high SLC and blood pressure when stratified on alleles of the α-adducin gene, suggesting heterogeneity in the SLC phenotype. If the SLC phenotype studied in the current analysis is composed of a combination of at least 2 subpopulations, stratification on α-adducin or other characteristics may be required to define a group with SLC that is strictly comparable to that studied in the inbred baboon population.

The demonstration of suggestive linkage between SLC and D15S642 requires confirmation in other white populations. Since ethnic differences are a prominent feature of SLC activity, studies in populations of African descent would be of considerable interest. Further efforts to identify candidate genes in the region identified on chromosome 15 should be possible with the use of SNP analysis of mapped genes.

**Perspectives**

Identifying genes underlying complex diseases such as hypertension with the use of traditional genetic approaches such as linkage analysis has proven difficult. In addition to the likelihood that each of the polygenes contributing to hypertension must perturb blood pressure homeostatic regulation only minimally, it is clear that the control of the set point of blood pressure regulation is rarely dependent on a single physiological mechanism, and each mechanism has its own set of genetic determinants. Even what to search for is problematic. Although mutations account for monogenic forms of hypertension, essential hypertension is a prototypic “disease of civilization.” It is the result of multiple gene-environment interactions in which “normal” genes are subjected to “abnormal,” ie, modern, environmental factors, the net effect of which is to raise blood pressure chronically. Quantifying “environment” is even more difficult than identifying genetic variants.

The present study suggests that the use of intermediate phenotypes closely associated with hypertension and highly heritable can increase the likelihood of discovering genetic determinants of hypertension. We are particularly intrigued by the finding that the peak we have identified as a QTL for SLC is at the same chromosome 15 marker previously identified as linked to blood pressure. Convergence of these 2 approaches provides strong evidence that the region is involved in the hypertensive process and should help guide the
identification of candidate genes involved in both blood pressure and SLC regulation.

Acknowledgments

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