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ARTICLE

Model-fitting and linkage analysis of sodium–lithium countertransport

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Increased sodium–lithium countertransport activity (SLC) associates with hypertension and is highly heritable, yet the underlying genes remain unknown. SLC, measured on 1113 and remeasured 2–3 years later on 675 adult members of 48 Utah pedigrees, was tested for candidate gene association, major locus inheritance, and linkage to genome scan markers using a bivariate model with genotype-specific effects of age, body mass index (BMI), and triglycerides level (TG). No effect of the α-adducin Gly460Trp polymorphism on SLC was found. In contrast, SLC increased with age in carriers of apolipoproteinE ε2 (85 individuals; 8.7% of the sample) and decreased in noncarriers. Model-fitting analyses inferred two additional loci with genotype-specific responses to BMI and TG. Using the inferred model, lod scores >2 were obtained for D3S3038, D11S4464, and D10S677 for the BMI-responsive locus, and for D8S1048 for the TG-responsive locus.

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Introduction

Increased sodium–lithium countertransport activity (SLC) in hypertensive patients was first reported over 20 years ago.[1] Many studies since support the hypertension association; in addition, hyperlipidemia and possibly diabetic nephropathy associate with increased SLC.[2]

SLC is highly heritable with heritability $\geqslant 65\%$ estimated in Utah pedigrees.[3,4] Nevertheless, no studied polymorphism affects SLC more than modestly (β_3-adrenergic receptor Trp64Arg,[5] α-adducin (ADD1) Gly460Trp[6,7]), while others are non-significant (Na-H antiporter,[8,9] angiotensin-converting enzyme insertion/deletion,[10,11] G-protein β_3-subunit C825T[12]) or inconsistent (blood group MNS,[10,13] haptoglobin,[10,13] apolipoproteinE (apoE)[14,15]).

Genome scans have implicated various genomic locations. Variance components linkage analysis applied to nuclear family data produced lod = 2.83 on chromosome 15q at marker D15S642[16] and applied to baboon pedigrees produced lod = 9.3 on chromosome 5, in the region homologous to human chromosome 4q near marker D4S1645.[17] Genome-wide association analysis of SLC in immortalized lymphoblasts identified multiple associations, many with markers near genes involved in glutathione metabolism.[18]

Herein, we analyzed SLC measured twice at a 2–3 years interval on members of 48 pedigrees. We tested for

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association with ADD1 and apoE polymorphisms, fit genetic models, and tested for linkage to genome-wide markers.

Subjects and methods

A total of 98 Utah pedigrees were ascertained through 2–3 siblings with stroke death before age 74, hypertensive and normotensive subjects from the Salt Lake Center of the Hypertension Detection and Followup Program, and men with coronary disease onset before age 55. Approximately 2500 members of the extended pedigrees were examined in 1980. Over 90% were re-examined in 1983. The study, described extensively elsewhere,[19] was approved by the Institutional Review Board of the University of Utah.

SLC was measured as the rate of sodium-dependent lithium efflux from lithium-loaded red blood cells using the method of Canessa *et al*[1] as adapted by Smith *et al*[20] Body mass index (BMI) was computed as the ratio of weight to height squared. Triglycerides level (TG) was measured using a coupled enzymatic method and commercial reagents. ApoE genotyping was performed on the LightCycler (Roche) using a commercial mutation detection kit (Roche) and rapid cycle real-time PCR; ADD1 genotyping was performed on the LightTyper (Roche) using a single fluorescent-labeled mutation detection probe (18-base synthetic oligonucleotide); postamplification fluorescent melting curve analysis followed both procedures.

The Mammalian Genotyping Service[21] genotyped 393 autosomal markers from Screening Set 9 on a sample subset comprising 1855 members of 49 pedigrees. Relationship and genotype incompatibilities were identified and corrected.[22] Mistyping analysis in Simwalk2,[23,24] using multipoint genotypes and files produced by MEGA2,[25] identified 1444 genotypes ($\sim$1.5% of the total) with error probability $\geqslant$25%, each henceforth designated as missing.

The analysis sample excluded members of ungenotyped pedigrees, individuals of age <18, women who were pregnant or taking oral contraceptives or hormone replacement therapy, and nine individuals with Exams 1–2 transformed SLC difference >0.25 since a large disparity suggested a measurement error. The analysis sample totaled 1113 individuals from 48 pedigrees ranging from 1 to 86 members, including 832 Exam 1 and 956 Exam 2 (675 overlap) measurements. SLC was square root transformed; BMI and TG were natural logarithm transformed.

We performed likelihood analysis of genetic models using Pedigree Analysis Package (PAP);[26] the mixed model likelihood was approximated.[27] No correction was made for ascertainment since most probands, being deceased, were unmeasured, and the pedigrees, extended to all available members, were not enriched for hypertension. We assumed a mixture of bivariate normal densities for Exams 1 and 2 SLC with mixture proportions determined by genotype frequencies in Hardy–Weinberg equilibrium. Univariate replaced bivariate densities for single-exam measurements. Parameters, assumed equal across exams, were estimated by maximizing the likelihood. Linear covariate effects were genotype-specific for age, BMI, and TG and genotype-constant for gender. The genotype-constant variance within major locus genotypes was partitioned between polygenes, a day-of-measurement effect shared within pedigrees, and random environmental effects. Setting the within-genotype genetic correlation $=1$ specified that the same polygenes affected Exams 1 and 2 SLC; the within-genotype environmental correlation was estimated. We tested significance of genotype-specific effects, by covariate and locus, using χ^2 statistics computed from likelihoods maximized assuming genotype-constant and genotype-specific effects.

We used measured genotype analysis to test whether ADD1 or apoE affected SLC by fixing each individual's genotype to his/her observed genotype in a one-locus version of the model described above. In each case, the sample was restricted to genotyped individuals.

The model-fitting analyses assumed the model described above with three unlinked loci, two alleles at each locus, and additivity across the loci.[28] One locus represented apoE by fixing each individual's genotype to his/her observed apoE genotype; apoE allele frequencies were fixed at estimates obtained from the sample assuming Hardy–Weinberg equilibrium and all familial relationships. The global maximum likelihood of the model was obtained through repeated searches of the likelihood surface starting from different sets of parameter values.

Linkage analysis of each marker to each of the two unidentified loci assumed the inferred three-locus model using PAP.[26] To speed computation, pedigree-specific lods included only informative marker alleles (producing a two-allele lod exceeding ±0.1 for recombination $=0.05$ for the designated allele *versus* all others) and multiallelic lods were computed only for promising markers (sum >0 of the two-allele lods across alleles and pedigrees) and for unpromising markers (sum <-1.5).

Results

Table 1 describes the analysis sample. A subset of 386 men and 289 women were measured twice at an average 2.4 years interval. The unadjusted Exams 1 and 2 SLC correlation equaled 0.753 ($P<0.0001$, $N=675$).

ADD1 Gly460Trp was genotyped on 359 sample members. The 460Trp allele frequency was estimated as 0.215, similar to control samples.[29,30] Genotype-specific effects were not significant for age ($\chi^2_{(1)}=3.38$, $P=0.18$), BMI ($\chi^2_{(1)}=4.26$, $P=0.12$), or TG ($\chi^2_{(1)}=0.98$, $P=0.61$). Assuming genotype-constant covariate effects, the genotype-specific means decreased nonsignificantly with the number of 460Trp alleles ($\chi^2_{(2)}=1.12$, $P=0.57$).

Table 1 Mean ± SD by exam and gender

	Exam 1		*Exam 2*	
Variable	*Men*	*Women*	*Men*	*Women*
Number	448	384	536	420
Number hypertensive	47	45	54	46
Age (years)	39.2 ± 13.8	39.9 ± 14.8	39.4 ± 14.5	40.9 ± 14.6
BMI (kg/m^2)	26.0 ± 3.9	25.6 ± 5.8	26.1 ± 4.2	26.1 ± 6.1
TG (mg/dl)	128 ± 87	101 ± 61	149 ± 102	110 ± 74
SLC (mmol/l RBC/h)	0.290 ± 0.111	0.257 ± 0.105	0.302 ± 0.108	0.261 ± 0.102

Table 2 *P*-values[a] for genotype-specific effects by locus, covariate, and sample

		Covariate		
Locus	*Sample*[b]	*Age*	*BMI*	*TG*
ApoE	Bivariate	0.025	0.14	0.63
	Univariate	0.17	0.11	0.63
BMI-responsive	Bivariate	0.58	0.030	0.76
	Univariate	0.85	0.054	0.94
TG-responsive	Bivariate	0.057	0.39	0.010
	Univariate	0.18	0.18	0.13

[a]For χ^2 statistic with df = 1 for apoE, df = 2 otherwise, testing genotype-constant *versus* genotype-specific effects of the covariates on SLC.
[b]The univariate sample was derived from the bivariate sample by excluding Exam 2 SLC whenever Exam 1 SLC was measured.

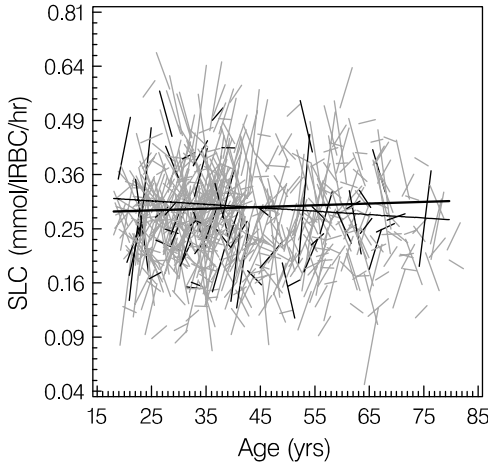

Figure 1 Genotype-specific age effects for apoE ε2 carriers (heavy line) and noncarriers (light line), assuming the most common genotypes at the BMI- and TG-responsive loci. Individual Exam 1 to Exam 2 changes with age in SLC adjusted for gender, BMI, and TG for ε2 carriers (short black lines) and noncarriers (gray lines) are presented.

ApoE was genotyped on 981 sample members. The frequencies of alleles ε2, ε3, and ε4 (0.056, 0.772, and 0.172, respectively) resemble estimates from comparable populations.[31] Because of small numbers, genotypes ε2/ε2 ($N = 2$) and ε2/ε4 ($N = 16$) were combined with ε2/ε3 ($N = 67$) and ε4/ε4 ($N = 21$) was combined with ε3/ε4 ($N = 282$). The effects of ε3 and ε4 did not differ ($\chi^2_{(4)} = 1.41$, $P = 0.84$), so further comparisons were limited to ε2 carriers ($N = 85$) and noncarriers. Genotype-specific effects on SLC were not significant for BMI ($\chi^2_{(1)} = 2.70$, $P = 0.10$) or TG ($\chi^2_{(1)} = 0.41$, $P = 0.52$), but were significant for age ($\chi^2_{(1)} = 5.37$, $P = 0.020$). Upon excluding the rare genotypes, we reached the same conclusions.

In addition to apoE, two unidentified loci affected SLC. Genotype-specific slopes differed significantly only for age for apoE, only for BMI at a second locus, and only for TG at a third locus (Table 2). None remained significant in univariate analysis after excluding repeat measurements (Table 2). The parsimonious model (all nonsignificant effects made genotype-constant) did not differ from the full model ($\chi^2_{(10)} = 10.39$, $P = 0.41$). Allele frequencies were estimated as 0.165 and 0.218 for the BMI- and TG-responsive loci, respectively. We partitioned the within-genotype variance as 50.4% to polygenes, 15.4% to measurement date, and the remaining 34.2% to random environmental effects. The within-genotype environmental correlation between exams was estimated as 15.9%.

Figure 1 shows individual Exam 1 to Exam 2 changes with age in SLC by ε2 carrier status. Annual rates of change, computed as Exams 1 and 2 transformed adjusted SLC difference divided by elapsed time, differed significantly between ε2 carriers and noncarriers ($t = 2.24$, $P = 0.026$). Figure 1 also shows the inferred slopes with age in ε2 carriers and noncarriers.

Figure 2 shows individual Exam 1 to Exam 2 changes with BMI in SLC. At the BMI-responsive locus, SLC increased least for common homozygotes (estimated as 73% of the population), most for rare homozygotes (2%), and intermediate for heterozygotes (25%).

Figure 3 shows individual Exam 1 to Exam 2 changes with TG in SLC. At the TG-responsive locus, SLC increased the least for heterozygotes (38%), intermediate for common homozygotes (55%), and the most for rare homo-

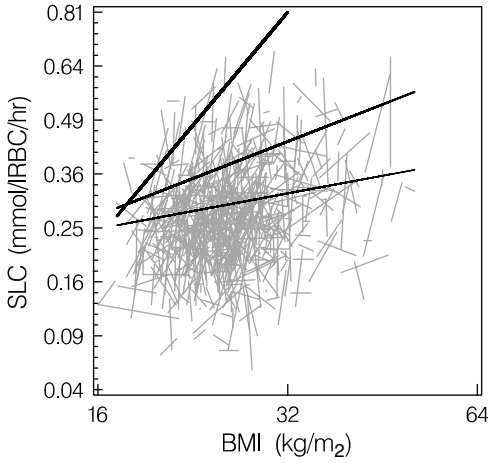

Figure 2 Genotype-specific BMI effects for rare homozygotes (heavy line), heterozygotes (medium line), and common homozygotes (light line) at the BMI-responsive locus, assuming the most common genotypes at the apoE- and TG-responsive loci. Individual Exam 1 to Exam 2 changes with BMI in SLC adjusted for gender, apoE genotype, and TG (gray lines) are presented.

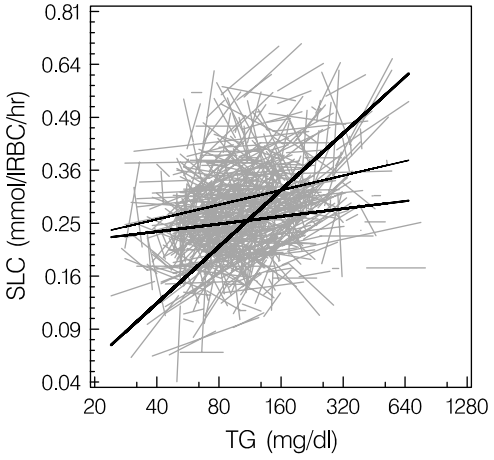

Figure 3 Genotype-specific TG effects for rare homozygotes (heavy line), heterozygotes (medium line), and common homozygotes (light line) at the TG-responsive locus, assuming the most common genotypes at apoE- and BMI-responsive loci. Individual Exam 1 to Exam 2 changes with TG in SLC adjusted for gender, apoE genotype, and BMI (gray lines) variables are shown.

zygotes (7%). Heterozygotes did not differ significantly from common homozygotes ($\chi^2_{(2)} = 3.18$, $P = 0.20$).

Tables3 and 4 present all lod scores >1.5 for the two inferred loci. For the BMI-responsive locus, 3 markers produced suggestive linkage evidence (lod >1.9[32]); for the TG-responsive locus, one marker produced suggestive linkage evidence; no significant linkage evidence (lod >3.3[32]) was obtained for either locus. The most highly negative lods obtained equaled -5.54 and -1.59 for the BMI- and TG-responsive loci, respectively, demonstrating substantially more power for the former than the latter.

Discussion

The inheritance of SLC appears very complex. This analysis suggests that, rather than acting directly, genes instead affect SLC through interaction with correlates; consequently, other genes acting on the correlates would indirectly affect SLC. BMI and TG are the strongest population-level correlates;[33–35] our model inferred an increase in SLC with BMI and TG at genotype-dependent rates. Although a weaker correlate, we found an interactive effect of age for apoE. We did not test for genotype-specific effects of other weaker correlates, such as HDL, glucose, and insulin.[33,34,36]

SLC was measured on our sample 20+ years ago. Since then, kinetic studies have shown that variation in activity reflects independent variation in V_{max} and k_m.[37] The hypertension association has been attributed to lower k_m,[38] whereas the hyperlipidemia association has been attributed to higher V_{max}.[37] Possibly, the BMI- and TG-responsive loci affect k_m and V_{max}, respectively.

The genotype-specific responsiveness of SLC to BMI or TG implies that weight loss or lipid reduction decreases SLC. In agreement, lipid reduction decreased SLC,[39–41] through decreased V_{max} with unchanged k_m.[41] However, SLC remained unchanged following weight loss accompanied by decreased TG, but the sample included only eight women.[42] Our model predicts a substantial response only in the subset of individuals with the responsive genotype.

The three major loci accounted for roughly 26% (apoE: 1%; BMI-responsive: 20%; TG-responsive: 5%) of the total or 40–45% of the genetic variance of SLC. Computational constraints prohibited attempting inference of a fourth locus. Possibly 10+ additional loci underlie the remaining 55–60% of the genetic variance, assuming each contributes less than the TG-responsive locus. The numerous marker associations[18] reported also support the presence of many genes.

ADD1 may be one of the additional genes, although, if so, we lacked sufficient power for confirmation. Other studies report that ADD1 affects SLC, but differ as to whether 460Trp decreases[7] or increases[6] SLC. Neither finding necessarily contradicts ours, since our model, although nonsignificant, estimates a steeper decrease with age and a steeper increase with BMI in 460Trp homozygotes compared to 460Gly homozygotes. Consequently, the ordering of the means across genotypes depends, as does the power, on the age and BMI profile of the sample. Another possibility, that ADD1 is the inferred BMI-responsive locus, is unlikely given the lack of evidence of linkage to markers on chromosome 4p16.3 near ADD1.

In contrast to ADD1, apoE attained significance; SLC increased with age in ε2 carriers and decreased in noncarriers. The small estimated difference in the slopes made significance surprising, but demonstrated the power inherent in measured, compared to unmeasured, genotypes. We estimated that ε2 carriers have lower SLC before and higher SLC after age 45. In disagreement, Wierzbicki *et al*[15] found ε2 associated with lower SLC, despite a mean age of 56. Stiefel *et al*[14] found no difference between ε4 carriers and noncarriers, in agreement with our results.

At the BMI-responsive locus, genotype affected SLC little in lean individuals, but increasingly with obesity. This model was not confirmed through significant linkage, but produced three suggestive linkages. One of these, D3S3038 on chromosome 3p21, produced a strong suggestive lod = 2.91. Two other markers, D11S4464 (lod = 2.45) and D15S655 (lod = 1.52), fall 2.1 and 2.8 cM, respectively, on the Marshfield map from D11S137 and D15S539, found to associate with SLC.[18]

At the TG-responsive locus, the rare homozygotes had the lowest SLC at low TG but the highest SLC at high TG, possibly because the linearity assumption is invalid. The TG-responsive locus produced only one suggestive linkage, to D8S1048, 4.6 cM on the LDB2000 map from the β_3-adrenergic receptor, for which SLC associates with Trp64Arg.[5] Although failing the suggestive linkage threshold, D10S1221 (lod = 1.75) and D8S2324 (lod = 1.55) fall on the Marshfield map <1 and 1.1 cM, respectively, from markers D10S122 and D8S1475 found to associate with SLC.[18]

Standard segregation analysis of SLC on this sample inferred major locus inheritance in only the pedigrees ascertained through coronary heart disease, but not stroke or hypertension,[4] motivating the inclusion herein of multiple loci to model genetic heterogeneity. In a previous attempt to dissect the heterogeneity, segregation analysis of principal component (PC) scores supported major locus inheritance of three scores to which SLC contributed substantially.[43] For PC1, homozygotes for a common allele showed higher SLC, BMI, and TG. For PC9, heterozygotes for a rare allele had higher SLC, but lower BMI. For PC11, homozygotes for a rare allele had higher SLC, but lower TG. Two-point model-based linkage analysis produced lod = 2.05 for PC1 with D3S3038 (Table 3), lod = 1.58 for SLC and lod = 1.54 for PC9 each with D1S160 (Table 4), and lod = 1.36 for PC9 with D1S1612 (Table 4). No other Table 3 or 4 markers produced lod > 1 and no other lod > 2 was obtained from two-point linkage analysis using the previously inferred models for SLC or the three scores.

Significant linkage evidence occasionally, but rarely, results when using models inferred from segregation analysis.[44,45] At fault may be the simplicity of standard segregation analysis models. In contrast, herein, we allowed for locus heterogeneity and genotype-specific covariate effects, and obtained higher lods than were obtained with the simpler model discussed above, and also higher lods than using variance components linkage analysis, which produced no lod > 2 for either Exam 1 or Exam 2 SLC (results not shown).

Table 3 Lod scores >1.5 for the BMI-responsive loci

Marker	*Chromosome*	*Location*[a]	*Lod*
D3S3038	3	45	2.91
D11S4464	11	123	2.45
D10S677	10	117	2.15
ATA58A02	17	107	1.57
D15S655	15	83	1.52

[a]cM from pter on the sex-averaged Marshfield map.

Table 4 Lod scores >1.5 for the TG-responsive loci

Marker	*Chromosome*	*Location*[a]	*Lod*
D8S1048	8	54	2.19
D10S1221	10	76	1.75
D1S160	1	0	1.75
D1S1612	1	16	1.67
D8S2324	8	94	1.55

[a]cM from pter on the sex-averaged Marshfield map.

In summary, we conclude that at least three genes affect SLC. For apoE, SLC increased significantly with age in ε2 carriers and decreased in noncarriers. In addition to apoE, we inferred that two unidentified loci, one showing a genotype-specific response to changes in BMI and the other showing a genotype-specific response to changes in TG. Lod scores >2 were obtained for markers D3S3038, D11S4464, and D10S677 for the BMI-responsive locus, and for D8S1048 for the TG-responsive locus.

Acknowledgements

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Electronic database information

URLs for data presented herein are as follows:
LDB2000 http://cedar.genetics.soton.ac.uk/public.html/LDB20001.html
Marshfield Linkage Maps, http://research.marshfieldclinic.org/genetics/
Mega2, http://watson.hgen.pitt.edu/mega2.html
PAP, http://hasstedt.utah.edu/
SimWalk2, http://www.genetics.ucla.edu/software/simwalk2.html

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