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ASSOCIATION OF *SLC34A2* VARIATION AND SODIUM-LITHIUM COUNTERTRANSPORT ACTIVITY IN HUMANS AND BABOONS

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Abstract

Background—Sodium lithium countertransport (SLC) activity, an intermediate phenotype of essential hypertension, has been linked to a region of baboon chromosome 5, homologous to human chromosome 4p. Human *SLC34A2*, located at chromosome 4p15.1-p15.3, is a positional candidate gene for SLC. The specific aim of this study was to identify genetic variants of the *SLC34A2* gene in both baboon and human, and to examine the relationship of these polymorphisms with SLC activity and blood pressure.

Methods—Single nucleotide polymorphism (SNP) was identified by sequencing the *SLC34A2* gene in 24 baboon founders and 94 unrelated individuals. All tag SNPs in *SLC34A2* were genotyped in 1856 individuals from 252 pedigrees of mixed European ancestry. Three SNPs in baboon were genotyped in 634 baboons comprising 11 pedigrees.

Results—In human, one SNP (rs12501856) was found to be significantly associated with SLC individually, though it did not pass multiple testing correction; however haplotype 2 containing allele C of SNP rs12501856 showed strong evidence of association with SLC (p=0.0037) after multiple comparison adjustment. This haplotype was also marginally associated with diastolic blood pressure and systolic blood pressure. This finding was confirmed in baboons, where a highly significant association was detected between SLC and baboon SNP *Asn136Asn* (p=0.0001). However, the associated SNP did not account for the linkage signal on baboon chromosome 5.

Conclusions—Consistent results in two different species imply that *SLC34A2* is associated with SLC activity and blood pressure.

INTRODUCTION

Essential hypertension, a major risk factor for cardiovascular diseases, is a multifactorial disease with 30~50% genetic contribution. 1, 2 It is well documented that essential hypertension is highly heterogeneous, individuals with the same blood pressure levels may have mutations at completely different loci, and also hypertension may involve the same

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disease loci with different alleles.3–5 This characteristic may explain the often conflicting results from genetic studies in essential hypertension. To use an intermediate trait which is predictive of essential hypertension and regulated by fewer genes and environmental factors, such as sodium lithium countertransport (SLC), may provide substantial advantages to gene discovery studies.

Sodium-lithium countertransport (SLC), first described by Canessa et al. 6, is assessed by measuring the rate of lithium loss from lithium loaded erythrocytes incubated in sodium-free versus sodium-rich medium. Canessa et al. 6 further reported that SLC is elevated in individuals with hypertension. SLC is a stable, bimodally distributed trait 7 with high estimated heritability (55%–88%) in both humans and baboons 8–10. There is strong evidence of a major gene influencing the distribution of SLC in humans 11–13. Turner and Michels 14 showed that there was significant correlation of SLC with blood pressure in the general population of Rochester, MN, and that this correlation persisted after adjusting for body mass index, triglycerides and total cholesterol among both men and women. Turner et al. 15 showed that for each standard deviation increase in SLC, the risk of hypertension approximately doubled in men (OR 2.25, 95% CI 1.44–3.51) and women (OR=1.77, 95% 1.32–2.37). Weder et al. 16 reported that adults with elevated SLC exhibited higher blood pressure levels as children, and elevated SLC in normotensive offspring of hypertensive parents has been reported in U.S. Caucasians, African Americans and Africans.17, 18 These observations suggest that SLC is a premorbid marker of essential hypertension in humans. Kammerer et al. 10 have presented convincing evidence of a locus influencing SLC on baboon chromosome 5, although the measurement of blood pressure in the unrestrained baboon is not technically feasible.

The SLC34 family of solute carriers comprises three members: Type II Na/P(i)-cotransporters NaPi-IIa (*SLC34A1*), NaPi-IIb (*SLC34A2*) and NaPi-IIc (*SLC34A3*). SLC34A2 cotransported phosphate and sodium into cells in the presence of sodium.19 It plays a role in sodium and phosphate homeostasis. Human *SLC34A2* is located at chromosome 4p15.1-p15.3 20 in a region of the genome homologous to the region of baboon chromosome 5, linked to SLC.10 Thus, *SLC34A2* is a positional and biological candidate gene for SLC. To examine the relationship between *SLC34A2* variation and SLC, we conducted a detailed sequence analysis of *SLC34A2* in baboons of known phenotype and of its' human ortholog, and conducted an association analysis between polymorphisms of *SLC34A2* and variation in SLC activity in 634 baboons comprising 11 pedigrees and 1856 individuals from 252 pedigrees of mixed European ancestry.

MATERIAL AND METHODS

Subjects

Individuals in this study were participants in the Rochester Family Heart Study (RFHS) phase II, including 252 pedigrees containing 1856 individuals. Multi-generation pedigrees were ascertained through households having two or more children enrolled in the school of Rochester, MN, between 1984 and 1991. The samples are of mixed European ancestry. The details of the recruitment have been reported previously.21, 22 All procedures were approved by the Institutional Review Board at the Mayo Clinic, Rochester, MN and all subjects gave written informed consent.

634 noninbred baboons (*Papio hamadryas*) comprising 11 pedigrees ranging in size from 16 to 99 animals. These 2- and 3-generation pedigrees consisted of 202 founders (29 sires and 173 dams) that were not selected for blood pressure and their 432 offspring. All experimental protocols were approved by the Southwest Foundation for Biomedical Research Institutional Animal Care and Use Committee.

Phenotyping

For humans, blood pressure was taken with a random zero sphygmomanometer (Howkeley and Sons LTD., West Sussex, UK) while subjects were seated in a quiet room. Average of three systolic readings and three diastolic readings for each subject were used in all analyses and are referred to as systolic and diastolic blood pressure.22 The prevalence of hypertension in this sample was 15.9% and 87.4% of these were receiving antihypertensive medications. These individuals were included in this analysis to be representatives of the population of Rochester. Blood pressure in baboons could not be measured except under chemical restraint and are not considered reliable for analysis. The maximal velocity of the SLC was used as an indicator of SLC activity and was determined by measuring the external sodium-stimulated lithium efflux from lithium-loaded RBCs as previously reported.6, 23 The coefficient of variation for assays done with fresh cells from the same individual on different days was 8.9%, and for replicate assays done on the same day was 7.5%. Details of the baboon phenotyping are presented in Kammerer et al. 10

Sequencing

Initial SNP identification was performed by sequencing each exon, exon-intron boundaries, and the proximal 5′ region, containing the putative promoter region, of *SLC34A2* in 94 unrelated individuals from RFHS phase II and 24 baboon founders. In order to maximize the potential genetic differences, those 94 subjects were selected from the highest and lowest deciles for SLC. The putative promoter region which was predicted by using Gene2Promotor program (Genomatix, Munich, Germany) is located around 500bp upstream of first coding exon (exon 2). Genomic DNA was isolated from peripheral blood leucocytes by standard procedures. DNA sequencing was performed by polymerase chain reaction (PCR) amplification (Supplement table 1 and 2) of the target fragment, purification of the product (ExoSAP-IT kit, USB corporation), and a sequencing using the ABI dRhodamine cycle sequencing kit (Applied Biosystems). Sequencing products were purified and applied to an ABI3700 capillary sequencer. Sequences were aligned and curated using the program SEQUENCHER (Gene Codes). All sequencing was carried out by the University of Pittsburgh, Genomics and Proteomics Core Laboratory (Pittsburgh, PA).

Genotyping

Human tag SNPs were selected using haploview 24 with data dumps from HapMap project, since the SNPs identified by resequencing are rare SNPs (MAF <0.05). All human tag SNPs with minor allele frequency (MAF) >0.05 were genotyped by Illumina Beadarray (Illumina Inc, San Diego, CA), genotypes of 4 SNPs rs12501856, rs6448389, rs3775909 and rs3796777 were further confirmed by fluorescence polarization (Supplement table 3) described by Chen *et al.*25, 26 using the L.J.L. Biosystems' Analyst HT Assay Detection System. Baboon SNPs were chosen on the basis of the sequencing results and genotyped by sequencing and fluorescence polarization. All potentially functional sites, missense mutations and variants located in functional domains with minor allele frequency ≥ 10%, were genotyped. Functional domains in *SLC34A2* were predicted using Bioinformatic Harvester.27 Since SNP *Lys636Asn* was in high Linkage Disequilibrium (LD) with *Leu630Leu* (r^2=0.98) and *Pro680Ala* (r^2=0.92), *Asn136Asn* was also in LD with *Glu61Glu* (r^2=0.78), we eventually chose to genotype three SNPs: *Lys636Asn*, *Asn136Asn* and *Lys645Glu* across the entire baboon population. R^2 was estimated by pair wise LD test in haploview software package.24

Data analysis—A basic X^2 goodness-of-fit test was used to test the deviations from the Hardy-Weinberg Equilibrium (HWE) in all genotype data from unrelated founders.

Mendelian error checking was performed using the INFER procedure in PEDSYS software version 2.0 (Southwest Foundation for Biomedical Research, San Antonio, TX).

Human SNP genotype association analyses were performed by SOLAR (Sequential Oligogenic Linkage Analysis Routines) Version 2.1.4.28 For each *SLC34A2* polymorphism, we used maximum likelihood methods to estimate the possible linear effects of each genotype on SLC activity. Models were also adjusted for age, sex and weight.10 Using the likelihood ratio test, we compared this model to a nested model in which the effects of the *SLC34A2* genotypes were set equal to zero. Associations were also confirmed by family-based association test (FBAT) version 1.5.1.29, 30 Option –e in FBAT, which could test for association in an area of known linkage, was used to complete the association analysis. 31 An additive model was tested. We used the Bonferroni correction to control for multiple testing. The haplotype version of FBAT (HBAT) was used to estimate the haplotype frequencies. Association of SLC and haplotypes having a ≥2% total frequency in the population ("major haplotypes") were further analyzed by HBAT.32

For baboon, we tested for an association between SLC activity and the *SLC34A2* polymorphisms using SOLAR. The adjusted means of SLC activity by SNP genotype were also estimated by SOLAR.33 Due to the complex family structure within the baboon pedigrees, we couldn't use FBAT and HBAT in association analysis for baboons. We derived haplotypes from baboon SNP genotypes using PHASE version 2.1 34, 35 and then incorporated the effects of each haplotype into our analyses. After detecting a significant effect of the *SLC34A2* SNP, variance component linkage analysis was used to evaluate whether this SNP account for the observed linkage signals on baboon chromosome 5.10 Variance component linkage analyses was performed by incorporating additive effects of the specific SNP genotype as a covariate, thus removing variation due to the *SLC34A2* SNP genotype. For details of the variance component linkage analysis see Kammerer et al.10 Multipoint linkage results of the model containing *SLC34A2* were compared to the original linkage results (that did not include *SLC34A2*). If the *SLC34A2* SNP is the sole functional polymorphisms accounting for the heritable variation of the trait, the linkage signal will completely disappear and the LOD score should drop to 0. If the measured SNP is only one of the several functional polymorphisms or is in disequilibrium with true variant, the evidence for linkage should remain.36

RESULTS

Characteristics of subjects

In humans, the number of males and females was almost equal and the average SLC activity was around 298.1 umol/l RBC/hr. For baboons, the total 204 males and 430 females had a mean SLC activity 242 umol/l RBC/hr. Detailed subject characteristics were shown in table 1.

Sequencing

Figure 1 summarizes the sequence variation identified in 94 humans and 24 baboons. A total of 5 exonic single nucleotide polymorphisms (SNP) and 10 intronic SNPs were observed in the human compared 17 exonic SNPs and 3 intronic SNPs to in the baboon, despite the smaller sample size of baboons. This difference in occurrence of SNPs between humans and baboons has been observed for other loci 37 and may be due to the mixed nature of the founding population of baboons, which included both *P.h. cynocephalus* and *P.h. anubis*, or to the difference in population history between humans and baboons.

Comparison of human and baboon nucleotide and amino acid sequence for *SLC34A2* is shown in Supplement Figure 1. Bioinformatic Harvester 27 predicted five domains,

including two sodium-phosphate (Na_Pi) cotransport domains, two low compositional complexity domains and one transmembrane domain which are highlighted in different colors in Supplement Figure 1. Low complexity regions are not well understood but have been shown to be functionally important in some proteins.38 Several baboon *SLC34A2* SNPs occur in regions important for *SLC34A2* function, for example, *Asn136Asn* (exon5) is located in Na_Pi cotransport domain and *Lys636Asn* (exon 13) is located in low compositional complexity domain. These SNPs as well as another missense mutation Lys645Glu were further genotyped in pedigreed baboons.

Genotyping

Supplement table 4 summarizes genotype and allele frequencies (based on 48 chromosomes) for humans. Five exonic and 15 intronic SNPs were observed.

Supplement table 5 summarizes genotype and allele frequencies observed in baboon samples. Seventeen exonic single nucleotide polymorphisms (SNP) and 3 intronic SNP were identified. Only a small region flanking each exon was sequenced so the number of intronic SNPs detected is small. No SNPs were shared between the two species.

Association analysis in humans

Of the seven *SLC34A2* SNPs genotyped in humans, SNP rs12501856 is associated with phenotypic variation in SLC (table 2). The p-values (0.037 for unadjusted and 0.024 for adjusted) by SOLAR are nominally significant, which was also confirmed by FBAT (P=0.03, data not shown), although it was not statistically significant after Bonferroni correction. However, haplotype association tests (table 3) show strong association (p=0.0027) between haplotype 2 in human *SLC34A2* and SLC activity which was significant after correction for multiple testing. Haplotype 2 was marginally significant association with SBP (p=0.073) and DBP (p=0.049). Mean SLC activity for the CC genotype was 317.2±11.3 μmol Li/l RBC/hr whereas for CT and TT individuals, mean SLC activity was around 288.6±5.1 μmol Li/l RBC/hr ($p<0.05$) (supplement table 6).

Linkage and association analysis in baboons

Among all genotyped sites in baboon, SNP *Asn136Asn* (exon5) shows (Table 4) strong evidence of association with SLC variation (p=0.0001) even after Bonferroni correction. The genotypes of this single SNP explained about 5% of total variance in SLC activity. The genotype specific effects on mean SLC activity for SNP Asn136Asn were similar as in human SNP rs12501856. The average SLC activity in individuals with CC genotype was much higher than ones in the other two groups (295.1±10.9 μmol Li/l RBC/hr, vs. 232.5±7.1 μmol Li/l RBC/hr, $p<0.01$). We also analyzed haplotypes at the SLC34A2 locus, and obtained a significant association with SLC activity (P= 0.0002). As expected, haplotypes containing the Asn136Asn C allele had increased SLC activity. (Data is not shown). In order to determine if *Asn136Asn* is the genetic variant that accounts for the baboon linkage signal identified by Kammerer et al,10 multipoint linkage analysis incorporating *Asn136Asn* genotypes was performed. The peak LOD score was only slightly reduced from 11.2 (in original linkage model that did not include *SLC34A2*) to 10.7 (in model containing *SLC34A2*) (data not shown), indicating this SNP did not account for the QTL (quantitative trait locus) effect.

DISCUSSION

Sodium lithium countertransport, a premorbid marker of essential hypertension, has been previously mapped to a region of baboon chromosome 5.10 Human *SLC34A2* is located at chromosome 4p15.1-p15.3 20 in a region of the genome homologous to the linkage region

of baboon chromosome 5, and near a suggestive linkage peak in humans making *SLC34A2* is a positional candidate gene for SLC activity.39

Resequencing of *SLC34A2* in the human and the baboon establishes the strong homology in exonic organization and sequence (96.7%) between the human and baboon *SLC34A2* genes and extensive variation in both species (Supplement Figure 1). In humans, the SNP rs12501856, located in intron 1 was significantly associated with increased SLC activity in single locus analysis (p=0.02), and with a more profound effect in haplotype analysis (p=0.004). This SNP differentiates h2 from the most frequent haplotype h1 and suggests that it is making variation in the 5′ end of *SLC34A2*. Unfortunately, the other haplotypes, h3-h10, occur at frequencies too low, ≤ 5%, to be more specific. Genotyping of SNPs in baboon *SLC34A2* revealed one variant of SNP *Asn136Asn* in exon 5 that is significantly (p=0.0001) associated with phenotypic variation in SLC activity. Baboons homozygous for the less common CC genotype had much higher levels of SLC activity than did carriers of the T allele. This SNP explained approximately 5% of the variation in SLC activity. It is worth noting that the baboon SNP which shows association with SLC is also in the 5′ region of the *SLC34A2* gene. There are many possible explanations for a difference in allelic effect between humans and baboon: 1) The environments of baboons are more homogeneous than those of humans, for example, baboons were fed the same diet and raised in similar housing. 2) General genetic and physiological differences between the two species. 3) Specific genotype effects of different SNPs in the two species.

To address the question if SNP *Asn136Asn* accounts for the linkage signal identified by Kammerer et al.,10 variance component linkage analysis which incorporated the measured genotype effect of this SNP into model was conducted. After removing the SNP effect, evidence of linkage remained in the model. There are several possible explanations for why this polymorphism showed strong association with SLC but minimally influenced the linkage signal. Firstly, because association analysis is far more powerful than linkage analysis in detecting the common variants with modest effects, it's possible that *Asn136Asn* is a surrogate for functional variation elsewhere in the gene. The most likely possibility is that another locus in linkage disequilibrium with gene *SLC34A2* explains these findings. It is also possible that the linkage signal is explained by several genes, *SLC34A2* being one of them but not the major gene influencing SLC activity. Lastly, the association is spurious; however, the consistent evidence of associations of SLC activity with *SLC34A2* in two different species makes the last explanation less likely. In addition, since our study design was family-based, including ascertainment of relatively large family-based samples and application of family-based association tests (FBAT), the effect of major confounding factor-population stratification in the association study was avoided. Also, the very small p-values of association tests in both species make the likelihood of false positive result very small.

Comparison of results in the human and baboon are limited by the fact that blood pressure in the baboon can not be measured except under chemical restraint leaving the relationship between hypertension and sodium lithium countertransport in the baboon open to question.

CONCLUSIONS

This study provides strong evidence that variation in *SLC34A2* is significantly associated with interindividual variation in sodium lithium countertransport in humans and baboons. In humans, a significantly higher SLC activity was associated with a haplotype marking the 5′ region of *SLC34A2*. In baboons, variation in *SLC34A2* explains a small but significant proportion of the variation in sodium lithium countertransport (≤ 5%) but is not the locus responsible for the strong evidence of linkage to chromosome 5 in the baboon. Thus,

SLC34A2 appears to be a relatively minor determinant of total sodium lithium countertransport, perhaps through linkage disequilibrium with major gene on baboon chromosome 5. The relationship of baboon chromosome 5 to the homologous region in the human genome is complicated by a complex inversion in this region in the baboon.

WEB RESOURCES

Accession numbers and URLs for data presented herein are as follows:

Bioinformatic Harvester, http://harvester.fzk.de/harvester/

Celera SNP Reference Database, http://www.celera.com/corporate/snpdata.html

CHIP Bioinformatics tools, http://snpper.chip.org/ (for the SNPper program)

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for *SLC34A2* cDNA sequence [accession number NM_006424], for genemic DNA sequence [accession number NC_000004]

Genomatix, http://www.genomatix.de/ (for the Gene2Promotor program)

Haploview, http://www.broad.mit.edu/mpg/haploview/

National Center for Health Statistics, http://www.cdc.gov/nchs/hus.htm

NCBI, http://www.ncbi.nlm.nih.gov/

University of Pittsburgh, Genomics and Proteomics core laboratory, http://www.genetics.pitt.edu

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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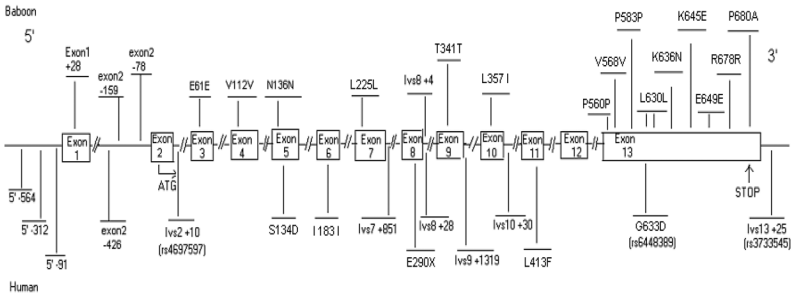

Figure 1. Baboon (top) and human (bottom) sequence variation in *SLC34A2*.

IVS: intervening sequence. For those SNPs previously reported for human, the rs# is given. All SNPs located in coding region were named in term of their amino acid locations. For SNPs located in non-coding region, the first base of any exon or IVS will be designed as "+1", any base in the upstream (closer to 5′) of it will be denoted as "−", and numbered from small to large according to their distance to "+1" from proximate to distal, e.g. the base next to it will be coded as "−1"; any base in the downstream (closer to 3′) of it will be denoted as "+".

Table 1

Characteristics of humans (RFHS II) and baboon used in this study

	Characteristics	Values*
RFHS II	Age (year)	41.1 ± 23.3
	Male (%)	51.1
	DBP(mmHg)	69.5 ± 11.1
	SBP(mmHg)	116.9 ± 20.2
	Height(cm)	165.9 ± 12.4
	Weight(kg)	68.7 ± 19.3
	BMI(kg/m^2)	24.6 ± 5.4
	SLC activity(umol/l RBC/hr)	298.1 ± 119.8
Baboon	Age (year)	9.4 ± 6.0
	Male (%)	32.2
	Weight(kg)	17.5±5.5
	SLC activity(μmol/l RBC/hr)	242±99

*Values are represented as mean ± SD (Standard Deviation)

Table 2

Results of human *SLC34A2* allelic association tests in Phase II

				SLC		SBP		DBP	
SNP	Position	MAF	HWE P	Unadjusted P	Adjusted P*	Unadjusted P	Adjusted P*	Unadjusted P	Adjusted P*
rs12501856	Intron 1	0.34	0.79	**0.04**	**0.02**	0.37	0.19	0.35	0.79
rs3775909	Intron 3	0.18	0.66	0.78	0.83	0.34	0.32	0.96	0.26
rs3796777	Intron 9	0.13	0.38	0.68	0.58	0.33	0.34	0.33	0.86
rs2240995	Intron 10	0.18	0.99	0.91	0.93	0.37	0.32	0.8	0.82
rs2240996	Intron 10	0.12	0.38	0.92	0.62	0.96	0.56	**0.037**	**0.07**
rs12505556	Intron 12	0.13	0.98	0.64	0.62	0.51	0.75	**0.038**	**0.05**
rs6448389	Exon13	0.13	0.27	0.12	0.39	0.98	0.44	0.52	0.74

SNP indicates single-nucleotide polymorphism; MAF, minor allele frequency; HWE, hardy-weinberg equilibrium; SLC, sodium lithium countertransport; SBP, systolic blood pressure; and DBP, diastolic blood pressure.

* P value is adjusted by age, sex and body mass index.

The significance level for a single test is set as p=0.007 (α = 0.05/7; Seven SNPs).

Table 3

Summary of haplotype association analysis results in human

Hap	Haplotype								SLC	SBP	DBP
No.	rs12501856	rs3775909	*rs3796777*	rs2240995	rs2240996	rs12505556	rs6448389	Freq	P value	P value	P value
h1	T	C	A	C	C	C	G	0.346	0.233	0.621	0.563
h2	C	C	A	C	C	C	G	0.159	**0.004**	**0.073**	**0.049**
h3	C	C	A	G	C	C	G	0.051	0.455	0.385	0.692
h4	T	G	G	C	C	C	G	0.043	0.659	0.431	0.428
h5	T	C	A	C	C	C	A	0.034	0.269	0.462	0.501
h6	T	C	A	G	C	C	G	0.03	0.786	0.68	0.6
h7	C	C	A	C	C	C	A	0.03	**0.023**	**0.027**	**0.029**
h8	C	C	A	C	A	C	G	0.03	0.717	0.324	0.378
h9	T	G	G	C	C	C	A	0.027	0.751	0.921	0.993
h10	T	G	A	C	A	C	G	0.022	0.558	0.652	0.622

Hap indicates haplotype; Freq, frequency.

The significance level for a single test is set as p=0.005 (α = 0.05/10; Ten haplotypes).

Table 4

Summary of results of baboon *SLC34A2* allelic association analyses

SNPs	Position	Minor Allele Frequency	HWE P value	SOLAR
Asn136Asn	exon 5	0.47	0.85	**0.0001**
Lys 636 Asn	exon 13	0.46	0.17	0.28
Lys 645 Glu	exon 13	0.1	0.14	0.27

The significance level for a single test is set as 0.0175 (α = 0.05/3; three SNPs).