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SEARCHING FOR GENETIC DETERMINANTS FOR SODIUM LITHIUM COUNTERTRANSPORT, AN INTERMEDIATE TRAIT FOR ESSENTIAL HYPERTENSION

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Essential hypertension (EH) is a major risk factor for cardiovascular disorders, the leading cause of death in the United States. Given its great public health impact, it is crucial to understand the genetic basis of EH. EH is highly heterogeneous and to use an intermediate phenotype of EH, sodium lithium countertransport (SLC), will provide substantial advantage for disease genes discovery. We proposed two approaches to explore the genes for SLC.

The first study examined the relationship between SLC and a positional candidate gene, *SLC34A2,* which is linked to SLC in baboon. We sequenced gene *SLC34A2* in baboon and human. Strong homology was established in exonic organization and sequence between human and baboon *SLC34A2* genes and extensive variation in both species was identified. Association studies between SLC and SLC34A2 were carried out in 1856 RFHS phase II individuals and 634 baboons. Significant association of SLC with human SNP rs3775909 ($p=0.03$) in *SLC34A2* and haplotype block 2 ($p<0.005$) were observed. Strong evidence for association of SLC with *SLC34A2* was found for baboon SNP Asn136Asn ($p=0.0001$). Consistent findings in two different species implied that *SLC34A2* may be one of the genes involved in SLC. However, linkage analyses conditional on genotypes of baboon Asn136Asn suggest that Asn136Asn is not the primarily functional site for SLC. We conclude that SLC34A2 is associated with SLC, though it may not be the major effect gene.

In second study, we integrated gene expression micrarray with linkage analysis to search for genes for SLC. Two independent microarrays (U133A and U133_plus_2.0) were used to identify the differentially expressed genes in high verse low SLC groups. Five genes, IER3, ARHGAP15, CD47, CDKAL1 and PRKRA, were among top 1% of differentially expressed genes in both arrays and also mapped to linkage region for SLC in RFHS Phase II population. A follow-up association study for IER3 shows that SNP rs8512 is significantly associated with SBP (p=0.002) and DBP (p=0.0008), and SNP rs2284174 has marginal association with SLC (p=0.055) and SBP (p=0.085). In conclusion, we identified some interesting susceptible genes for SLC by combining gene expression profiling and linkage study.

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1.0 BACKGROUND AND SIGNIFICANCE

1.1 ESSENTIAL HYPERTENSION

1.1.1 Epidemiology and genetics of essential hypertension

Hypertension is a common vascular disorder, defined as occurring when the systolic pressure is persistently over 140 mm Hg, or the diastolic blood pressure is consistently over 90 mm Hg (Chobanian et al. 2003). Essential hypertension which refers to hypertension without known detectable etiology, accounts for 94% of all hypertension. Over 60 million people in United States were affected by essential hypertension (National center for health statistic, [CDC], 2005). It is also the most frequent reason that Americans visit a doctor. Essential hypertension is generally asymptomatic, occationally causing headache, but it is a major risk factor for stroke, myocardial infarction and end-stage renal disease, all of which are leading causes of death and illness in the United States. The yearly disease-related spending is almost $108.8 billion (Hodgson et al. 2001). Given its great public health impact, it is crucial to understand the etiology of essential hypertension.

Essential hypertension is one of the most complex disorders. It’s determined by environmental, genetic and demographic factors as well as their interactions (Hamet et al. 1998; Marenberg et al. 1994; Douglas et al. 1996). Twin and family aggregation studies have suggested

that the estimated genetic contribution to hypertension is around 30%-50% (Ward 1990; Snieder et al. 2003). Although the genetic basis for several rare monogenic forms of hypertension has been well established, these rare mutations in single genes are unlikely to determine all the variation in blood pressure in the population at large. The reasons for this assumption derive from the nature of this disorder. Blood pressure control is a polygenic phenotype involving a complex combination of processes and systems (Ferrannini 1991). It is regulated by numerous traits with balancing depressor and pressor roles, and the effect of any single gene may be muted due to this functional redundancy. Province et al. (2003) further reported that multiple genes involved in hypertension might have only small to moderate effects; no major genes with large effect existed (Thiel et al. 2003). These features of essential hypertension have confounded the study of the genetic etiology of this disorder. The substantial heterogeneity due to the gene-environment and gene-gene interactions makes it even more elusive. It is well documented that essential hypertension is highly heterogeneous (Laragh and Pecker 1983; Resnick and Laragh 1985; Mullins et al. 1996; Kurtz et al. 1989; Tournoy et al. 1996). Individuals with the same blood pressure levels may have mutations at completely different loci, or hypertension may involve the same disease loci with different alleles.

1.1.2 Genetic studies for essential hypertension

Intensive efforts have been taken to characterize the genes involved in essential hypertension. At present most published data arise from candidate gene studies and genome-wide scans, but contradictory results have been achieved in these studies. Candidate genes, based on their biochemical or physiological features which are likely to participate in blood pressure regulation, may be selected from chromosome loci identified by linkage mapping or congenic

analysis. Differences in allele frequencies at marker loci of candidate gene in unrelated case and control individuals are tested by association study. True association appears when the marker itself contributes to hypertension or alleles at marker locus are in linkage disequilibrium (the frequency of the presence of two alleles in one gamete in a population is higher than would be expected from random recombination) with the disease causative allele. To date, more than 150 candidate genes have been proposed to influence blood pressure (Kato et al. 1998; Tao et al. 1995; Markovic et al. 2005; Quintana et al. 2006), including genes coding vascular proteins, metabolic proteins, adrenoceptors, proteins involved in renin-angiotensin-aldosterone system and volume and/or sodium homeostasis.

None of the genes is consistently associated with essential hypertension in general population. The non-replication might result from different genetic background in various populations, the relatively small effect of a single gene, genetic heterogeneity of essential hypertension, or spurious association derived from multiple tests or population stratification. Population stratification is a confounding factor in case-control association studies. It occurs when allele frequencies are different in cases and controls due to ethnic substructure, and the disease prevalence between the two ethnic groups is dissimilar. For example, if two ancestral populations with different disease prevalence happen to be over-represented in either cases or controls, and if the allele frequency of a marker is different between the two populations, a false positive association may appear. To reduce the effect of population stratification, a family based study design, including transmission disequilibrium test (TDT) has been suggested in recently studies (Laird et al. 2004; Laird 2000; Rabinowitz and Laird 2000; Horvath et al. 2001). TDT only uses genotype data from parents and their offspring, and tests whether the probability of transmission of a marker allele from affected heterozygous parents to an affected offspring is

more than 50%. The hypothesis (H_A) of the test is that the marker is in both linkage and association with disease loci. If the marker is associated but not linked to the disease loci, they won't tend to be transmitted together, therefore no allele will be transmitted to affected offspring more than expected; if the marker is linked but not associated with the disease loci, the transmitted alleles will be different in different families. Similarly overall no marker will have more possibility than chance to be transmitted to affected kids. This feature of the TDT potentially avoids the spurious association due to population stratification. However, due to the difficulty in collecting large family-based samples, it has not been widely used in genetic studies yet.

Genome-wide linkage analysis once dominated the gene discovery for hypertension in the middle of the 1990's. However, linkage and association studies are not mutually exclusive approaches. Linkage analysis examines the co-segregation with hypertension of genetic markers within families. The assumption is that the genetic marker tested is linked to disease causative gene. Usually linkage study is used to map the susceptible genes into a region around 10-30 CM (10-30 Mb); an association study then refines the map to identify the target gene. Suggestive linkage regions have been identified for essential hypertension on multiple human chromosomes, including chromosomes 1, 2, 3, 4, 5, 6, 10, 11, 15, 16, 17, 19, and 22 (Rice et al. 2000; Perola et al. 2000; Krushkal et al. 1999; Levy et al. 2000; Sharma et al. 2000; Xu et al. 1999; Andrea et al. 2002, Caulfield et al. 2003), which also confirmed that essential hypertension is a polygenetic disorder. With the availability of high throughput genotyping and the completion of Hapmap project, genome-wide association has been employed in studies of essential hypertension in recent years (Nakayama et al., 2004). An association study has greater power than linkage to identify genes of small effect, but it is also more liable to show false positive result due to

genetic conversion, and variation in the pattern of linkage disequilibrium cross human genome. When the disequilibrium between the disease locus and marker is low, or there is allelic heterogeneity, linkage analysis might be a better approach.

1.1.3 Statement of the problem and solution

A common obstacle for both linkage and association studies is the heterogeneity of essential hypertension, which might explain the conflicting observations found in genome-wide scans. Individuals having the genetic predisposition to hypertension may not necessarily develop hypertension, and individuals may have hypertension due to non-genetic factors. The use of the intermediate trait previously associated with hypertension and one that may be regulated by fewer genes and environmental factors, such as sodium-lithium countertransport, provide substantial advantages for using either linkage or association tests. It can improve the power of linkage analysis, because the intermediate phenotype is closer than hypertension to the proximate gene product and is influenced by less confounding factors, it's more likely to follow the classical Mendelian pattern of inheritance and make the genetic signal stronger and more detectable. For an association study, intermediate traits can help with identifying true cases and controls which is essential to find the disease causing genes. Unfortunately, not many studies have yet used the intermediate phenotype in exploiting the etiology of essential hypertension.

1.2 SODIUM LITHIUM COUNTERTRANSPORT

1.2.1 Genetics of sodium lithium countertransport

Sodium-lithium countertransport (SLC), a membrane transport phenotype first reported by Canessa et al. (1980), is assessed by measuring the rate of lithium loss from lithium loaded erythrocytes incubated in sodium-free versus sodium-rich medium. Canessa et al. (1980) further reported that SLC is elevated in individuals with hypertension. Since that, increased SLC has been consistently documented in individuals with essential hypertension. Turner et al. (1992) 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. (1991) reported that adults with elevated SLC exhibited higher blood pressure levels as children. Elevated SLC in normotensive offspring of hypertensive parents has also been observed in U.S. Caucasians, African Americans and Africans (Woods et al. 1982; Obasohan et al. 1998).

The reported heritability of SLC is fairly high in both humans and baboons (58%-88%) (Hasstedt et al. 1988; Dadone et al. 1984; Kammerer et al. 2001). However little is known about its genetic etiology. Turner et al. (1985) reported that SLC is a stable, bimodal distributed quantitative trait. Although the bimodality itself is not direct evidence for the existence of a major genetic determinant for the SLC, it implies the possibility. Later, complex segregation analyses (Boerwinkle et al. 1986; Rebbeck et al. 1991; Motulsky et al. 1987) presented strong evidence that a single gene with large effects might contribute to the distribution of SLC in the population at large. These findings were supported by study of Rebbeck et al.(1993), who further proposed that a biometrically inferred major gene might be used to predict the development of

hypertension, since the odds of having hypertension increased 5.2 times more in men with this statistically inferred “high” SLC genotype compared with those with alternative genotypes. Similar results were also achieved in a prospective study of adults from Utah pedigrees (Hunt et al. 1991; Williams et al. 1994). A large polygenic contribution to the variation in SLC has also been postulated (Hasstedt et al. 1994; Schork et al. 2002; Weder et al 2003; Dadone et al. 1984).

The observations of strong association of SLC with essential hypertension, the high heritability of SLC in human and other species as well as the potential existence of single gene or genes with large effect on SLC, suggest that SLC may be a good intermediate trait for essential hypertension.

1.2.2 Functions of sodium lithium countertransport

The role of sodium lithium countertransport in the pathology of essential hypertension is unknown. SLC activity has typically been measured in mature erythrocytes; however if SLC activity can only been observed in erythrocytes, its function in essential hypertension would be doubted. Grinstein et al. (1984) first reported an SLC-like activity in thymic lymphocytes, then Zerbini et al. (1997 and 2001) found SLC activity was significantly elevated in human skin fibroblast from hypertensive individuals, extended the association of SLC with essential hypertension to cells outside erythrocyte, which also potentially made the involvement of SLC in development of human essential hypertension possible.

Several hypotheses were proposed regarding what catalyzed the exchange (West et al. 1998), but none of them has been validated.

Model I

The role of sodium balance (Dahl 2005; Boero et al. 1989; Hasegawa et al. 1987; Weinberger and Fineberg 2001) in the development of hypertension has been very well established. It was originally assumed that SLC may represent a mode of operation of some abnormal membrane sodium transport (Hardman et al. 1999; Guarena, et al. 1993; Brearley et al. 1993; Hunt et al. 2006; Meneton et al. 2001) which may cause essential hypertension. For example, Na^+/H^+ exchanger (NHE) was reported to be correlated with SLC (Touyz et al. 1999; Bradford et al. 1989; Ng et al. 2000; Zerbini et al. 2003) by a presumable mechanism of altered smooth muscle growth and contraction, but there're debates on that (Zerbini et al. 1998; van Norren et al. 1997; Lifton et al, 1991).

Model II

The observations that SLC activity is elevated in both essential hypertension and diabetes patients (Grunfeld et al. 1994; Houtman et al. 1995; Andronico et al. 1998; Monciotti et al. 1997) and the Na^+/H^+ exchanger was activated by insulin receptor kinase in hepatocytes (Demaurex and Grinstein 1994, Bianchini and Poussegur 1994) triggered a hypothesis. It was assumed that the membrane sodium transport itself might not cause hypertension, alteration of SLC activity and essential hypertension were equally caused by raised insulin, which might mediate SLC through activating some membrane transport or a mechanism involving the cytoskeleton. However, contradictory results of the relationship between SLC and insulin have been reported (Grunfeld et al. 1994; Herlitz et al. 2001; Foyle and Drury 1991; Rutherford et al. 1993; Canessa et al. 1992; Canessa 1994; Zerbini et al. 1995)

Model III

Abnormalities in modification of SLC by alkylation of erythrocyte thiol groups have been found to be very similar in patients with hypertension, diabetes and diabetic nephropathy

(Vareesangthip et al. 1996; Thomas et al. 1995; Vareesangthip et al. 2006). Therefore, a hypothesis that the aberrent thiol modification (e.g. oxidation) of cell membrane or cytoskeletal components may be resposible for these diseases was proposed. However, no solid evidence has been shown to support this hypothesis yet.

Model IV

It has been acknowledged that hyperlipidemia is involved in development of essential hypertension. The association of SLC with hyperlipidemia has been reported by several studies (Wierzbicki et al. 2001; Wierzbicki et al. 2000; Macleond et al. 1997; Engelmann et al. 1993). This prompts a hypothesis that the change of SLC kinetics may be caused by hyperlipidemia through a mechanism of altering the erythrocyte membrane viscosity. However, the fact that association of SLC with hypertension remains even after adjustment of blood lipid levels suggests that this mechanism alone can not explain the relationship between SLC and hypertension.

1.2.3 Significance of characterizing genetic variants determining SLC

Sodium lithium countertransport has been well established as an intermediate marker for essential hypertension, but little is known about its genetic determinants. Searching for the genetic variants influencing sodium lithium countertransport will lead to a better understanding of how alteration of SLC activity may contribute to the elevated blood pressure and its clinical complications, and eventually to a better understanding of the etiology of essential hypertension. It may also help with early diagnosis of young normotensive individuals with predisposition to hypertension and to better treatment options.

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2.0 A POSITIONAL CANDIDATE GENE FOR SODIUM LITHIUM COUNTERTRANSPORT-SLC34A2

2.1 SPECIFIC AIM

To examine the relationship between a positional candidate gene-*SLC34A2* (Type IIb sodium dependent phosphate co-transporter) and erythrocyte SLC in both baboon and human.

2.2 BACKGROUND

2.2.1 Baboon model

Baboons (*Papio hamadryas*) are among the closest living relatives to humans in terms of evolution and genetics. They share many genetic, metabolic and physiological features with humans, which make them excellent animal models for hypertension.

Kammerer et al. (2001) have studied the genetic and environmental factors that affect erythrocyte SLC in 634 baboons comprising 11 pedigrees and presented convincing evidence of a locus influencing SLC on baboon chromosome 5.

2.2.2 Positional candidate gene-*SLC34A2*

SLC34A2 (Type IIb sodium dependent phosphate co-transporter) belongs to the SLC34 family of solute carriers, which comprise three members: Type II Na/P(i)-cotransporters NaPi-IIa (*SLC34A1*), NaPi-IIb (*SLC34A2*) and NaPi-IIc (*SLC34A3*). The major role of this family is thought to maintain phosphate homeostasis (Murer et al., 2004). Hattenhauer et al. reported that NaPi-llb is involved in sodium dependent transcellular flux of phosphate in the small intestine. (Hattenhauer et al, 1999; Hilfiker et al, 1998; Katai et al, 1999)**.** The role of *SLC34A2* in other tissues has yet to be well defined.

Human SCL34A2 is located at chromosome 4p15.1-p15.3 (Xu et al. 1999) in a region of the genome homologous to the region of baboon chromosome 5, linked to SLC (Kammerer et al., 2001). Thus, *SLC34A2* is a positional candidate gene for SLC.

2.3 EXPERIMENTAL DESIGN

This specific aim was accomplished through the completion of the following steps:

- Sequencing *SLC34A2* in 24 founders from 11 baboon pedigrees and 94 individuals from Rochester Family Heart Study (RFHS) phase II.
- Comparing structural variants between two species.
- Genotyping most informative SNPs across the *SLC34A2* locus in 634 baboons, and 1856 individuals from RFHS phase II.
- Evaluating the relationships between the allelic variation of SNPs and the variation of SLC activity by linkage and association tests.

2.4 MATERIAL AND METHODS

2.4.1 Human Samples - Rochester Family Heart Study

The objective of the Rochester Family Heart Study (RFHS) is to identify genetic variation that influences the risk of cardiovascular disease in the population of Rochester, MN. Multi-generation pedigrees were ascertained through households having two or more children enrolled in the school of Rochester between 1984 and 1991. Members were ascertained regardless of health status. The samples are of mixed European ancestry. The sampling details, clinic examination protocol, and baseline features have been described by Moll et al (1983) and Turner et al (1989). Clinical and laboratory data and DNA samples from the members of RFHS phase II, including 252 pedigrees containing 1856 individuals were used to accomplish the specific aim of project 1 with informed consent. Confidentiality of all subjects participated the study is fully protected. The characteristics of individuals in the RFHS phase II are shown in table 1.

The average SBP and DBP were 116.9 and 69.5 mmHg respectively, which fall into the normal range of blood pressure level. The rate of males and females is almost equal. The average SLC activity was 298.1umol/l RBC/hr.

Table 1 Characteristics of individuals in the RFHS phase II

Characteristics	RFHS Phase 2 (n=1856)
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

2.4.2 Animal samples – baboons

Blood samples from 634 noninbred baboons (*P. hamadryas*) comprising 11 pedigrees of 2 and 3 generations each were used for measuring the SLC activity and genotyping. 24 baboons out of approximately 202 founders in these pedigrees were selected for DNA sequencing (Kammerer et al, 2001). All experimental protocols were approved by the Southwest Foundation institutional animal care and use committee. Table 2 shows the characteristics of 634 baboons.

Table 2 Characteristics of baboons

Characteristics	Values
Age (year)	9.4 ± 6.0
Sex (F/M)	430/204
Weight(kg)	17.5±5.5
SLC activity(umol/l RBC/hr)	0.242±0.099

2.4.3 Measurement of SLC activities

Data on baboon and human SLC activity were supplied by Dr. Kammerer and Dr. Morrison respectively. SLC is assessed by measuring the rate of lithium loss from lithium loaded erythrocytes incubated in sodium-free versus sodium-rich medium.

In brief, 20 to 30 mL blood samples were drawn from the femoral vein. The RBCs were separated from plasma and buffy coat by centrifuging at 1000*g* for 10 minutes. After being washed three times with a washing solution (150 mmol/l choline chloride, 1 mmol/l $MgCl_2$, 10 mmol/L glucose, 10 mmol/l Tris-MOPS), 5 mL of packed RBCs was removed and used for sodium lithium countertransport assay. The SLC activity assay measures the external Na^+-stimulated Li^+ efflux by comparing the lithium loss rates from lithium loaded erythrocytes incubated in sodium-free verse sodium-rich medium. The method had been previously described by Canessa et al. (1980) and Smith et al. (1984). Briefly, RBCs were suspended and incubated in 20 mL 150 mmol/L LiCl solution for 3 hours at 37°C for lithium loading. Samples were then incubated with sodium-containing media of 150 mmol/L NaCl, 10 mmol/L ouabain, 10 mmol/L

glucose, and 10 mmol/L Tris-MOPS (pH 7.4 at 37°C) or with sodium-free solution of 150 mmol/L choline chloride, 10 mmol/L ouabain, 10 mmol/L glucose, and 10 mmol/L Tris-MOPS (pH 7.4 at 37°C). Lithium concentration was measured at different time points (0, 30, 50, 70, and 90 minutes) by atomic absorption spectroscopy. The erythrocytes lithium efflux rate was calculated by linear regression analysis of sample time versus lithium concentration. Samples with the linear regression $r^2 < 0.9$ were removed from further analyses. The difference between the lithium efflux rates of RBCs incubated in sodium-containing media and sodium-free solution is the maximal velocity of SLC, which is expressed as micromoles lithium per liter RBCs per hour. Blind duplicates of RBC samples were used for quality control. (Modified from Kammerer et al., 2001)

2.4.4 Taqman real time RT-PCR

Total RNA from human tissues (kidney, arota, heart, left atrium, left ventricle, placenta, liver, lung, brain, skeletal muscle) was purchased from Ambion, Inc. (Austin, TX). Taqman Assays-on-demand for human *SLC34A2* (including primers and probe) and Taqman GAPDH Control reagents were also from Ambion, Inc. Taqman RT−PCR assay was conducted according to the manufacturer's specifications (Applied Biosystems, Forest City, CA). In brief, for each tissue, RNA dilutions of 80 and 20ng/µl were made in Nuclease-free water and used for two separate RT reactions. cDNA was synthesized using TaqMan RT kit (Applied Biosystems, Forest City, CA), and the 100ul RT reaction mixtures (contained 5ul RNA dilutions) were incubated in a thermal cycler with 10 min at 25°C, 45 min at 48°C, heat inactivated for 5 min at 95°C, hold at 4°C. 5ul of each RT sample was used in the Taqman PCR reaction and all reactions (*SLC34A2* and GAPDH were in separate tubes) were carried out in 96-well plates using

the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) under the following conditions: 95°C for 12 min, and 40 cycles of 95°C for 15 sec and 60°C for 60 sec. Each sample was done in triplicate. Reactions in which reverse transcriptase was not added were used to control for DNA contamination. The comparative C_T method was used to calculate the relative expression of *SLC34A2* mRNA in different tissues according to the manufacturer's protocol (Applied Biosystems). The tissue with the lowest expression level was used as a calibrator. C_t values of both the calibrator and the samples of interest are normalized to an endogenous housekeeping gene - GAPDH. The relative expression level was calculated by an arithmetic formula $2^{-\Delta\Delta CT}$.

2.4.5 Prediction of promoter sequences

Promoter sequences of *SLC34A2* were predicted by using Gene2Promoter in Genomatix software (Genomatix Software GmbH, Munich, Germany).

2.4.6 Sequence analysis of *SLC34A2*

Genomic DNA was isolated from peripheral blood leucocytes (Baboon and human DNA were supplied by Dr. Cox, Southwest Foundation and Dr. Morrison, University of Texas respectively). DNA from 24 baboon founders and 94 individuals with extreme (top 15 percentile vs. bottom 15 percentile) of SLC levels (Fig. 1) belonging to RFHS phase II was used for our sequence analysis. To obtain sufficient DNA, samples from 24 baboon founders were whole genome amplified using the method of Dean et al. (2002) and the Genomiphi (Amersham Biosciences) whole genome amplification protocol. Briefly, 1 µL of DNA was added to 9 µL of

sample buffer containing random hexamers and heated to 95°C to denature the DNA. The sample was cooled and mixed with 9 µL of reaction buffer containing salts and deoxynucleotides and 1 µL of enzyme mix. The mixture was incubated overnight at 30°C. After amplification, the Phi29 DNA polymerase was heat-inactivated during a 10 minute incubation at 65°C.

Each exon, exon-intron boundary, proximal 5' region and predicted promoter region of *SLC34A2* was thoroughly sequenced. Unique sequence primers for amplifying *SLC34A2* exons and flanking regions were designed using the human genomic sequence (Genbank NT 006316.15) and synthesized by DNA Synthesis Facility at University of Pittsburgh. PCR amplifications were carried out by using Taq DNA polymerase kit (Invitrogen Corporation, CA). 50 ng of DNA was added to a reaction mixture, including 10pmoles of each of primer pair (Table 3), 10nmoles of each dNTP, 10xbuffer 5ul, Mg2+ 2ul (Table 3), Tag DNA polymerase 1U. Adjust the final total reaction volume to 50 µl by adding deionized water. Samples were denatured at 95°C for 3 min. followed by cycles (indicated in table 3 and table 4) of denaturation at 95°C for 30 sec., annealing at temperatures indicated in Table 3 and table 4 for 30 sec. and extension at 72°C for 1 min. 8%(v/v, final concentration) betaine and dimethyl sulfoxide (DMSO) (Promega Corp., Madison, WI) was added in PCR mixture during amplification of human exon 1. A two-step PCR was used to specifically amplify baboon exon 1, because two DNA fragments were amplified in the first step. After the primary PCR, products were separated on 2% agarose gel and the fragment with the exact molecular size of exon 1 was extracted from agarose gels according to Nucleospin extraction kits protocols (BD Biosciences Clontech, CA). The reisolated PCR fragment was used as a template for secondary PCR reamplification. All amplimers (10ul) were electrophoresed in 1% agarose, stained with ethidium bromide and visualized under UV illumination to confirm the size and purity of the product.

The ExoSAP process was used to clean up the PCR products before sequencing. Briefly, we incubate 5ul PCR product with 5ul master mix of ExoSAP (0.5ul shrimp alkaline phosphatase, 0.05ul of exonuclease I, 0.5ul buffer and 4ul deionized water) at 37°C for 30 min., followed by denaturation at 88°C for 15 min.

Amplimers were sequenced from both directions using the ABI dRhodamine cycle sequencing kit (Applied Biosystems, Forest City, CA) as described in protocol. Mix 2.5ul PCR product, 2.3ul primer (0.8uM), 0.575ul dRhodamine dye and 1.725ul of sequencing buffer together. Incubate reaction mixture at 96 ^{o}C for 10sec., 50 ^{o}C for 5 sec. and 60 ^{o}C for 4 min., repeated by 24 cycles. Products were cleaned by Cleanseq Dye-Terminator Removal kit (Agencourt Bioscience Corporation, Beverly, MA). Briefly, add 10ul of clean sequence beads and 38ul of 85% ethanol to each sample, vortex for 30 sec., then place in magnetic tray for 10 min. aspirate the cleared supernatant. Dispense 100ul of 85% ethanol and incubate at room temperature for 30 sec. aspirate out the ethanol, air dry for 10 min. Elute the sequencing products from the beads by elution buffer and load onto the ABI3700 capillary sequencer. Sequences were aligned and curated using the program SEQUENCHER (Gene Codes).

2.4.7 Functional Domain Prediction

Functional domains in *SLC34A2* were predicted by using Bioinformatic Harvester (Urban Liebel and Bjoern Kindler, the European Molecular Biology Laboratory - Heidelberg, Germany) (Liebel et al 2005), a bioinformatic meta search engine. It can provide comprehensive gene-protein information by searching 16 major databases and prediction servers and combining the results.

Table 3 Amplication and Sequencing reaction components and conditions for Baboon *SLC34A2*

Amplified region		Primers (5'-3') Baboon	Positions	Annealing temp	Mg2+ (mM)	Cycles
Exon 1	1F	AGCCTCTTTGCGCAACAC	5' -55 ~ -38	59°C	1	36
	1R	AGTCAGCACCGAGAGGTA	IVS1 +73 ~ +90			
Predicted promoter	F	GAGATTAAAGGTGGTACATGCAGCTA	Exon2 -281~ -256	60°C	1.5	36
	R	CGTGGAGTAGGACGGCAGAAGTTCAA	Exon2 +234~ +259			
Exon 2	2F	CCTCCTTTCCATGACTGC	exon2 -45~ -28	58.5°C	1	35
	2R	TCACAAAGGCCGATCTGC	IVS2 +22~ +39			
Exon 3	3F	GTAAGTGTCGCTCGTTTG	IVS2 +1~ +18	56°C	1	38
	3R	TGACACCGTGAACCACAG	IVS3 +42~ +59			
Exon 4	4F	GAGCTCTGAGCTCATTGC	exon4 -91~ -74	58.5°C	1	35
	4R	ACACTCCAGGAGGCTGCA	IVS +90~ +107			
Exon 5	5F	CCCTCGATCACGTTGTGA	exon5 -49~ -32	53°C	1.25	36
	5R	TAGACAGGAGAGTGTCTG	IVS5 +35~ +52			
Exon 6	6F	CATAGTGAGGTGCAGAGT	exon6 -157~ -140	56°C	1	36
	6R	CAATAGAGCTGATA*TCGC	IVS6 +25~ +42			
Exon 7	7F	GTACATGTGCACACTTCC	exon7 -126~ -109	58°C	1	35
	7R	CCAGGCCCAGAATAGTGT	IVS7 +88~+105			
Exon 8	8F	CAGTGCCTCTGCAGATAG	exon8 -157~ -140	58°C	1	35
	8R	AGGCATTCACTGGTCACT	IVS8 +132~ +149			
Exon 9	9F	GTGTCTGCGCCTGTTCAT	exon9 -92~ -75	57.5°C	1	35
	9R	CCAGACAACACCTGTCAT	IVS9 +29~ +46			
Exon 10	10F	GTCTTGATTTGGGGCTGC	exon10 -140~-123	58°C	0.75	35
	10R	AGAGGCCATTGCTAGATG	IVS10+73~ +90			
Exon 11	11F	TGATGTACAACCTCACCC	exon11 -98~ -81	58°C	1	35
	11R	CAGCGTGAGTGGATAAGC	exon12 +27~ +44			

Table 3 continued

Amplified region		Primers (5'-3') Baboon	Positions	Annealing temp	Mg2+ (mM)	Cycles
Exon 12	12F	CTGCCATTTCCTGTCATC	exon12 -57~ -40	57.5°C	1.25	35
	12R	GGCTCTTGTTAGAGACCA	IVS12 +63~ +80			
Exon 13	13F1	CTCACCTGTCCAACCTCT	exon13 -28~ -11	59°C	1	38
	13R1	CAAGTCCTT*GCAGCACTT	exon13 +469~+486			
Exon 13	13F2	CGTCGTCTCCAAGTTCAC	exon13 +357~+374	53°C	1	38
	13R2	GTTAGGTAGGACTGCATC	IVS13 +123~ +140			

*This nucleotide is different in human *SLC34A2*. In human sequence, it's G instead of A at exon 6; it's C instead of T at exon 13.
F and R: Forward and Reverse primers for PCR amplification respectively.

Table 4 Amplication and Sequencing reaction conditions for human *SLC34A2*

Amplified region		Primers (5'-3') Human	Positions	Annealing temp	Mg2+ (mM)	Cycles
Exon 1	1F	CTTAGGACGGAGTGGTGA	5' -581~ -598	55.5°C	0.9	36
	1R	TGAAGTCAGCACCGAGAG	IVS1 +76~ +93			
Predicted	F_1	GCAATGGCACAATCTCAGCTCACTGC	Exon2 -575~ -550	64°C	2	36
Promoter	R_1	CTAAGTGTGGCATATTAAGCACCAT	Exon2 -185~ -161			
Predicted	F_2	GAGATTAAAGGTGGTACATGCAGCTA	Exon2 -281~ -256	60°C	1.5	36
Promoter	R_2	CGTGGAGTAGGACGGCAGAAGTTCAA	Exon2 +234~ +259			
Exon 2	2F	CACCCAGTTGATGCTTTG	exon2 -100~ -83	58°C	1	35
	2R	CTATCAGTGTAGCCGTGG	exon3 +52~+69			
Exon 3	3F	GTAAGTGTCGCTCGTTTG	IVS2 +1~ +18	58°C	1.25	35
	3R	GGTGACAGAGCAAGACTC	IVS3 +157~ +174			
Exon 4	4F	CTGTAAGGCTGGCTAGAC	exon4 -156~ -139	58°C	1	35
	4R	CCTAAAACACTCCAGGAG	IVS4 +96~ +113			

Table 4 continued

Amplified region		Primers (5'-3') Human	Positions	Annealing temp	Mg2+ (mM)	Cycles
Exon 5	5F 5R	TGGACTCTGCAACCCACA GCCTGGACATATTCAGAG	exon5 -118~ -101 IVS5 +85~ +102	58°C	1	36
Exon 6	6F 6R	CATAGTGAGGTGCAGAGT GCTGGTGAGTATGTGATC	exon6 -157~ -140 IVS6 +152~ +169	58°C	1	35
Exon 7	7F 7R	GTACATGTGCACACTTCC CCAGGCCCAGAATAGTGT	exon7 -126~ -109 IVS7 +88~+105	58°C	1	35
Exon 8	8F 8R	CAGTGCCTCTGCAGATAG AGGCATTCACTGGTCACT	exon8 -157~ -140 IVS8 +132~ +149	58°C	1	35
Exon 9	9F 9R	GCCATACTGCATGCACCA GTTGCTCACTGCTTGATG	exon9 -116~ -99 IVS9 +122~ +139	58°C	0.75	35
Exon 10	10F 10R	GTCTTGATTTGGGGCTGC AGAGGCCATTGCTAGATG	exon10 -140~-123 IVS10+73~ +90	58°C	0.75	35
Exon 11	11F 11R	TGATGTACAACCTCACCC CAGCGTGAGTGGATAAGC	exon11 -98~ -81 exon12 +27~ +44	58°C	1	35
Exon 12	12F 12R	GTACAGAGCAGCTCTGTG GGTCTTACTGCTTCTCTC	exon11 +72~ +89 IVS112 +187~+204	58°C	1	35
Exon 13	$13F_1$ $13R_1$	TGATGCCTGCTAGCTTAC GTCTCAGGAGCCTTGACA	exon13 -92~ -75 exon13 +516~ +533	58°C	1	36
Exon 13	$13F_2$ $13R_2$	CGTCGTCTCCAAGTTCAC GTACACTTCCTCATCTGG	exon13 +357~ +374 IVS13 +403~ +420	58°C	0.75	36

2.4.8 Genotypes of *SLC34A2* in baboon and human

2.4.8.1 Selection of SNPs

Informative SNPs of human *SLC34A2* were genotyped in 1856 individuals from RFHS phase II. Human SNPs for genotyping were selected based on our resequencing results, a private database Celera and the public database SNPper (CHIP Bioinformatics Tools, Riva and Kohane, 2002). All genotyped SNPs had a minor allele frequency $\geq$10%.

For association and linkage analysis, all 634 baboons were genotyped. Baboon *SLC34A2* SNPs were chosen based on our sequencing data with the help of Haploview. The functional sites such as missense mutations and mutations located in important functional domains with minor allele frequency $\geq$10% were genotyped.

2.4.8.2 High throughput genotyping - TDI-FP

All genotyping was done by TDI-FP (Template direct dye-terminator incorporation with fluorescence-polarization assay), except for SNPs in baboon exon 13, which were done by sequencing by Dr. Laura A. Cox. The reason is that several informative SNPs are located on the same exon, so it is more efficient to genotype these SNPs by one time sequencing.

PCR amplifications were carried out in a total reaction volume of 20 µl, containing 10ng of baboon DNA (due to limited amount of available baboon DNA) or 25ng of human DNA, 4 pmoles of each primer (Table 3), 4 nmoles of each dNTP, 2.0ul of 10xbuffer, 0.8ul of Mg2+ (Table 3) and 0.9U Tag DNA polymerase. The amplification and amplicon check procedures were the same as described before. Excess PCR primers and unincorporated dNTPs were degraded by ExoSAP process (USB Corporation, Cleveland, Ohio, USA). Incubate 10ul PCR product with 10ul master mix of ExoSAP (1ul shrimp alkaline phosphatase, 0.1ul of exonuclease

I, 1ul buffer and 8ul deionized water) at 37°C for 90 min., followed by denaturation at 95°C for 15 min.

A template direct dye-terminator incorporation with fluorescence-polarization (TDI-FP) assay as previously described (Chen et al, 1999; Kwok 2002) was then performed for high throughput genotyping. The cleaned PCR product (total volume 20ul) was combined with 0.05 µl thermosequenase DNA polymerase(USB corporation, OH), 1.0 µl 10X reaction buffer, 0.05 µl of the 1:16 two-dye mix (eg. for SNP C/T, 16ul 0.1mM ddATP, 16ul 0.1mM ddGTP, 15ul 0.1mM ddCTP, 16ul 0.1mM ddTTP, 1ul R110-UTP, 1ul TAMRA-CTP) (Perkin Elmer Life Sciences, Inc, Boston, MA), 1.0 µl 10 µmol/L SNP specific primer (DNA Synthesis Facility, University of Pittsburgh,PA), and 8 µl water. Template-directed incorporation (TDI) was performed in 96-well, black-skirted plates (MJ Research, Waltham, MA) using a Tetrad thermal cycler (MJ Research) under the condition at 95°C for 1 min, followed by 35 cycles of 94°C for 10 s and annealing temperature (see table 4) for 30 s with a final hold step of 4°C. FP was measured by L.J.L biosystem's Analyst HT Assay Detection System and analyzed by Allele Caller software package (L.J.L biosystems, Sunnyvale, CA). The amplification and TDI-FP primers and conditions were summarized in Table 5.

Table 5 Amplification and FP primers and conditions for genotyping tag SNPs of *SLC34A2* in human and baboons

SNPs ID	Celera ID	rs #	position		Primers	Annealing temp	Mg2+ (mM)
*SLC34A2*_1 Human	cv1222755	rs12501856	Intron 1	F	CTTGTGATGGCTGTAAGG	54°C	1.5
				R	CATAGGCACCACAACACT		
				FP-F	GAATTTTTGAAGCACAGAGTCA	55°C	
				FP-R	AAAACTTTCTTGTACTTTGAAAGA		
*SLC34A2*_2 Human	cv1222758	rs3775909	Intron 3	F	TATGCAGCCAGACAGCTG	56°C	2
				R	CCACAGTGTCACTGAGTG		
				FP-F	AGCCAGTGGAGTAAACAGCA	55°C	
				FP-R	CCTGGGATGCTACATCCTTAA		
*SLC34A2*_3 Human	cv122770	rs3796777	Intron 9	F	AACCCAGCCATCCTTCGTTATTC	59°C	1.5
				R	GATAATCCCAGCAAAACATGG		
				FP-F	CCACATTAATTCTGAGCACTATA	55°C	
				FP-R	AATCATACCTAGAGTATTAAG		
*SLC34A2*_4 Human	By resequencing	Gly 633 Asp	exon 13	F	CTCCAGAACTGGAACTTC	54°C	2
				R	CAAGTCCTCGCAGCACTT		
				FP-F	GCGTGCTGCTTGCTGTGTG	55°C	
				FP-R	CGGCAGCACTTGGGGCAG		
Baboon	By sequencing	Asn 136 Asn	exon5	F	CCCTCGATCACGTTGTGA	53°C	1.25
				R	TAGACAGGAGAGTGTCTG		
				FP-F	GCAGGACAGTTCTTCAGCAA	52°C	
				FP-R	AGGGTTGGACATAATAGAGCT		

2.5 DATA ANALYSIS

2.5.1 Tests of Hardy-Weinberg Equilibrium

A basic X^2 goodness-of-fit test was used to test the deviations from the Hardy-Weinberg Equilibrium (HWE). We didn't conduct HWE test for sequencing data in baboon due to the small sample size (only 24 samples).

2.5.2 Linkage and association analysis

2.5.2.1 Baboon

Allelic association analyses for baboon *SLC34A2* were conducted by the variance component method in SOLAR (Sequential Oligogenic Linkage Analysis Routines) Version 2.1.4 (Southwest Foundation for Biomedical Research, San Antonio, TX) that was extended for use on full pedigrees (Almasy and Blangero, 1998).Genotype-data cleaning, including Mendelian error checking, was performed using the INFER procedure in PEDSYS software version 2.0 (Southwest Foundation for Biomedical Research, San Antonio, TX). An additive model was used for all analyses. The measured genotypes were recoded as covariates - most common homozygous genotype was coded as 0, the heterozygous and the less common homozygous genotype were coded as 1 and 2 respectively. Their effects on the trait were then estimated by the maximum likelihood ratio test through comparing the model including the covariate against the model excluding the covariate by SOLAR. The other estimated covariates included age, sex and weight. The Bonferroni correction was used for the multiple testing adjustments.

For the SNP significantly associated with the trait, the means of the trait in each measured genotype were calculated by SOLAR to further understand the genotype specific effect on the means of the trait. In this case, three genotypes AA (common genotype), AB, BB were recoded as dummy variables 0 0, 0 1 and 1 0 respectively. The means of trait for each genotype group were calculated by the formula: mean + ß*1; mean is the final trait mean in the optimized regression model, ß is the slope rate of each genotypic covariate, which were reported in Poly.out file in SOLAR.

To evaluate the attribution of a specific *SLC34A2* SNP to the observed SLC linkage signals by Kammerer (2001), linkage analysis conditional on measured genotype was performed. Highly polymorphic microsatellite markers data which was used to localize a quantitative trait locus (QTL) for baboon SLC was obtained from Dr. Candace Kammerer. The multipoint variance component linkage analyses was conducted by calculating the $\log_{10}$ likelihood ratio (LOD) of a polygenic model that does not incorporate genetic marker information and models that incorporate marker genotype data across a chromosome (Levy et al. 2000) by using SOLAR. Linkage was reevaluated conditional on the genotypes of interest. Multipoint IBD probabilities were generated by CRIMP linkage analysis package along chromosome 4 at 1 cM intervals. The measured genotypes were modeled as a covariate with fixed effect on the trait mean. By this way, the variance due to this SNP was removed from the final linkage model. The contrast between the original and conditional LODs was used to assess the attribution of a SNP to the linkage component. If the SNP is the sole functional variant accounted for the trait, the linkage signal will completely disappear and the LOD score should drop to 0 in the conditional linkage analysis. 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 in the conditional analysis (Almasy and Blangero, 2004).

(All statistical tests in baboon were carried out with the collaboration of Dr. Candace Kammerer and Marget Kenney).

2.5.2.2 Human

Human SNP genotype association analyses were performed by both SOLAR and family-based association test (FBAT) version 1.5.1 (Harvard school of public health, Boston, MA) (Laird et al. 2000 and Horvath et al. 2001). The procedure for data cleaning was same as described for the baboon. The null hypothesis of FBAT is “no linkage and association” between the marker and the disease underlying locus. Option –e in FBAT, which could test for association in an area of known linkage (Lake et al., 2000) was used to complete the association analysis. An additive model was tested. The haplotype version of FBAT (HBAT) was used to estimate the associations between SLC and SNPs hyplotypes (Horvath et al. 2004). Due to the complex family structure within the baboon pedigrees, we couldn’t use FBAT in association analysis for baboons.

The association analysis in SOLAR was similar to that described in baboon, except for the estimation of covariates. The effects of covariates, including age, sex, height, weight, BMI, Cholesterol, LDL, HDL, fasting, triglycerides and smoke status were first assessed by SAS (SAS, version 9.1, SAS institute Inc., Cary, NC). Those with P values less than 0.1 were re-examined by SOLAR, and covariates which didn’t significantly influence the trait were removed from the final model. The results were adjusted for multiple testing using the Bonferroni correction.

(All statistical analyses in human were done with the collaboration of Dr. Alanna Morrison, Human Gentic Center, Houston, TX).

2.6 RESULTS

2.6.1 The distribution histogram for sodium lithium countertransport

The frequency distribution histogram for sodium lithium countertransport in human RFHS phase II is shown in Figure 1. The distribution is skewed toward higher values, which is consistent with previous report (Turner et al. 1985). Forty seven individuals from the top 15th percentile and forty seven individuals from the lower 15th percentile of the distribution were used for sequencing.

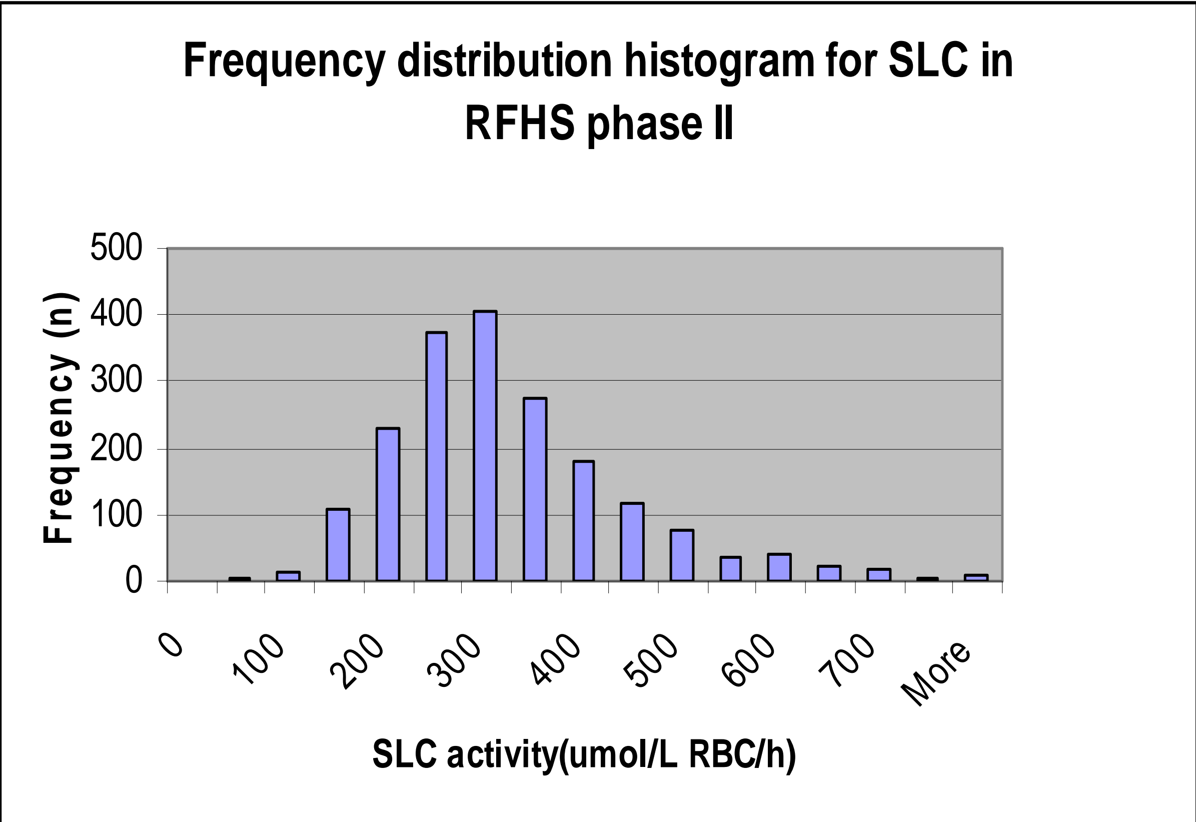

Figure 1 The frequency distribution histogram for sodium lithium countertransport in human RFHS phase II

2.6.2 Relative expression of *SLC34A2* in human tissues

It is shown in figure 2 that *SLC34A2* has high expression in lung, kidney, placenta and liver; moderate in aorta, left atrium and heart; lowest in skeletal muscle.

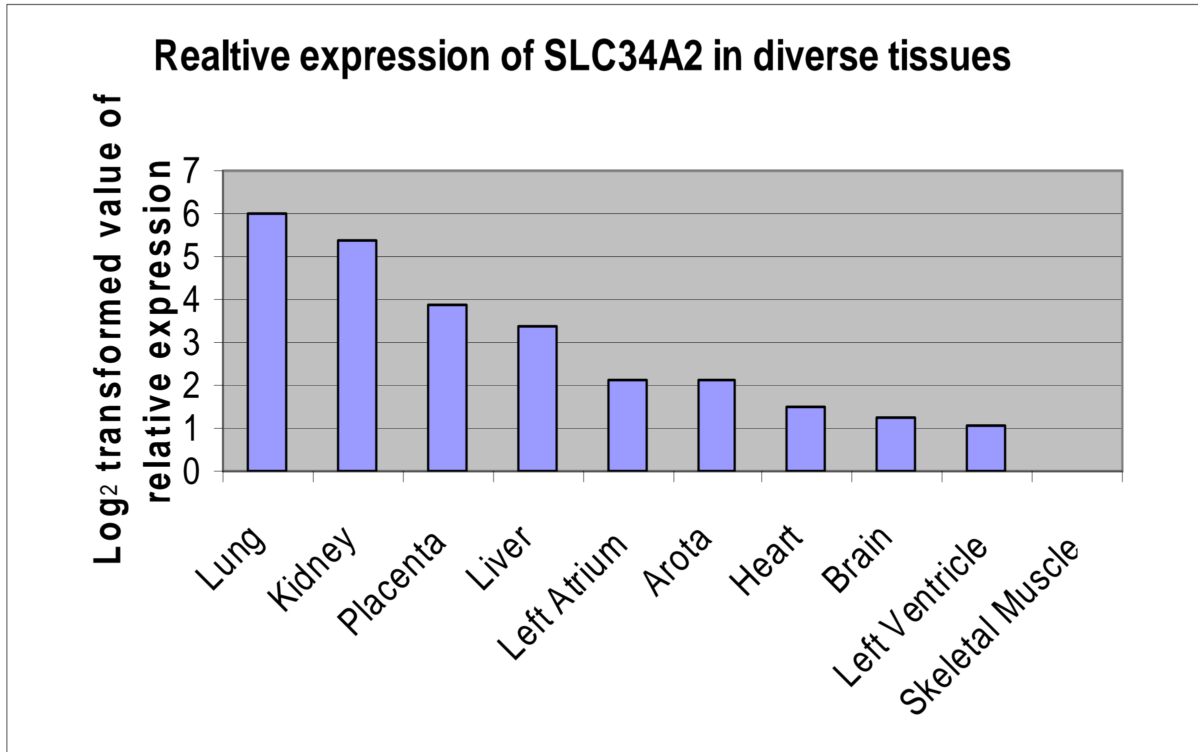

Figure 2 Relative Expression of *SLC34A2* in diverse tissues

Figure 2 shows relative expression of *SLC34A2* in human tissues with potential functions in blood pressure regulation. The RNA from skeletal muscle was used as calibrator. Y-axis indicates the relative expression values in diverse tissues expressed as log2 transformed $2^{-[delta][delta]Ct}$.

2.6.3 The sequence variation observed in the baboon and in human

Figure 3 summarizes the sequence variation observed in 24 baboons and 94 humans. The Genomatix predicted promoter region in human genome sequence starts from exon2 -503 and ends at exon2 +92. A total of 16 exonic single nucleotide polymorphisms (SNP) and 3 intronic SNPswere observed in the baboon compared to 5 exonic SNPs and 10 intronic SNPs in the human, despite the smaller sample size. This difference in occurrence of SNP variation between humans and baboons has been observed for other loci (Wang et al. 2004) 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.

The comparison of the nucleotide and amino acid sequence alignments of *SLC34A2* between human and baboon is shown in appendix 1. Bioinformatic Harvester (Liebel et al 2005) found five predicted domains, including 2 sodium-phosphate (Na_Pi) cotransport domains, 2 low compositional complexity domains and 1 transmembrane domain which were highlighted in different colors in appendix 1. "Low complexity regions are regions of biased composition. Theses regions are often mosiacs of a small number of amino acids. These regions have been shown to be functionally important in some proteins, but they are generally not very well understood". This description is from Pfam: help page (Wan et al. 2000 and Wootton 1994). There are several SNPs in baboon which occur in regions important for *SLC34A2* function. For example, the SNP Asn136Asn (exon5) located in Na_Pi cotransport domains and Lys636Asn (exon 13) in low compositional complexity domains are very interesting SNPs. These SNPs as well as other missense mutations with minor allele frequency ≥10% were genotyped in all baboon pedigrees.

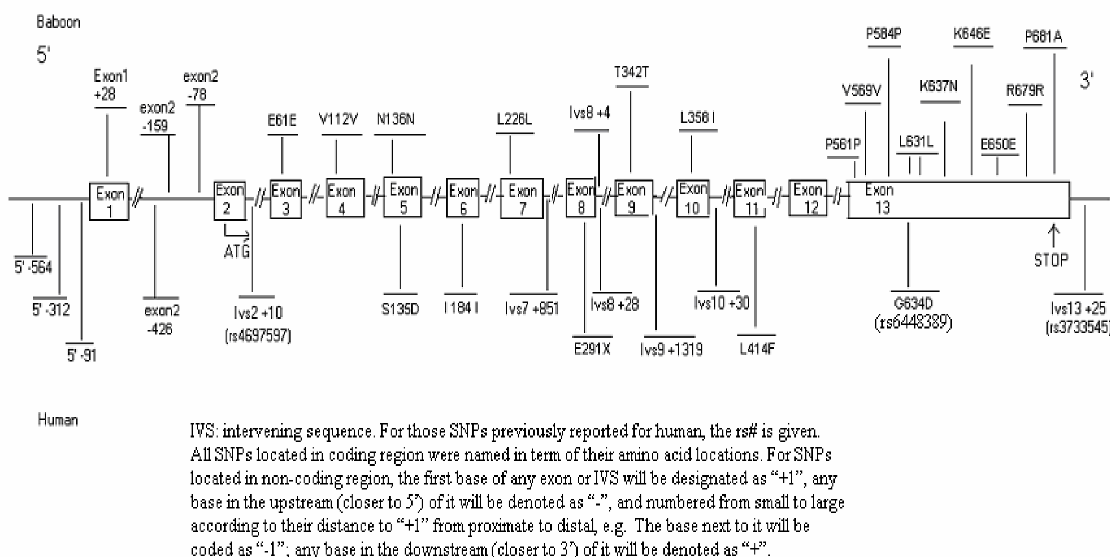

Figure 3 Baboon and human sequence variation in SLC34A2

2.6.4 Single nucleotide polymorphisms identified in sequencing SLC34A2 in 24 baboons

Table 6 summarizes the genotype and allele frequencies (based on 48 chromosomes) observed in this sample. Appearing to depart from Hardy Weinberg Equilibrium for SNP Asn136Asn is due to small sample size and the fact that the 24 founders used for sequencing were not a random sample of the population. Follow-up genotyping Asn136Asn in all 634 baboon pedigrees showed that it didn't deviate from Hardy Weinberg Equilibrium at all (p=0.85). Only a small region flanking each exon was sequenced so the number of intronic SNP

detected is small. Table 7 summarizes genotype and allele frequencies for humans. No SNP was shared between the two species.

Table 6 Single nucleotide polymorphisms identified in sequencing SLC34A2 in 24 baboons

Region	SNP	Genotypes #		Allele freq
Exon 1	Exon1 +28	G/G	22	G = 0.958
(5' UTR)	(G-->A)	G/A	2	A = 0.042
Promoter	Exon2 -159	G/G	20	G = 0.927
		G/A	4	A = 0.083
Promoter	Exon2 -78	T/T	20	T = 0.927
		T/C	4	C = 0.083
Exon 3	Glu 61 Glu	G/G	13	G = 0.708
	(G-->A)	G/A	8	A = 0.292
		A/A	3	
Exon 4	Val 112 Val	G/G	23	G = 0.979
	(G-->A)	G/A	1	A = 0.021
Exon 5	Asn 136 Asn *	T/T	12	T = 0.604
	(T-->C)	T/C	5	C = 0.396
		C/C	7	
Exon 7	Leu 225 Leu	C/C	23	C = 0.979
	(C-->G)	G/C	1	G = 0.021
intron 8	IVS 8 +4	C/C	22	C = 0.958
	C-->T	C/T	2	T = 0.042
Exon 9	Thr 341 Thr	C/C	21	C = 0.917
	(C-->T)	T/C	2	T = 0.083
		T/T	1	
Exon 10	Leu 357 Ile	C/C	22	C = 0.958
	(C-->A)	A/C	2	A = 0.042

Table 6 continued

Region	SNP	Genotypes #		Allele freq
Exon 13	Pro 560 Pro	C/C	19	C = 0.875
	(C-->T)	C/T	4	T = 0.125
		T/T	1	
	Val 568 Val	A/A	23	A = 0.958
	(A-->T)	T/T	1	T = 0.042
	Pro 583 Pro	C/C	23	C = 0.979
	(C-->G)	G/C	1	G = 0.021
	Leu 630 Leu	T/T	7	T = 0.521
	(T-->C)	T/C	11	C = 0.479
		C/C	6	
	Leu 630 Leu	G/G	21	G = 0.938
	(G-->A)	A/G	3	A = 0.062
	Lys 636 Asn *	G/G	6	G = 0.479
	(G-->C)	G/C	11	C = 0.521
		C/C	7	
	Lys 645 Glu *	A/A	21	A = 0.896
	(A-->C)	C/A	3	C = 0.104
		C/C	1	
	Glu 649 Glu	G/G	21	G = 0.938
	(G-->A)	A/G	3	A = 0.062
	Arg 678 Arg	T/T	23	T = 0.958
	(T-->G)	G/G	1	G = 0.042
	Pro 680 Ala	C/C	6	C = 0.5
	(C-->G)	G/C	12	G = 0.5
		G/G	6	

* SNPs were further genotyped. ** SNPs are in LD with Lys 636 Asn.

Table 7 Single nucleotide polymorphisms identified in sequencing *SLC34A2* in 94 unrelated individuals from RFHS Phase II

Sequencing region	SNP	Genotypes	#	Allele frequencies	HWE P value
5' region	5' -564 (T-->C)	T/T T/C C/C	69 8 1	T = 0.936 C = 0.064	0.63
	5' -312 (G-->C)	G/G G/C	72 12	G = 0.929 C = 0.071	1
	5' -91 (G-->T)	G/G G/T	79 4	G = 0.976 T = 0.024	1
Promoter	Exon2 -426 (C-->T)	C/C C/T	88 4	C = 0.978 T = 0.022	1
Intron 2	IVS2 +10 (rs4697597) (G-->A)	G/G G/A	90 4	G = 0.979 A = 0.021	1
Exon 5	Ser134Asp (G-->A)	G/G G/A	91 1	G = 0.995 A = 0.005	1
Exon 6	Iso 183 Iso (T-->C)	T/T T/C	88 4	T = 0.978 C = 0.022	1
Intron 7	IVS7 +851 (G-->A)	G/G G/A	91 1	G = 0.995 A = 0.005	1
Exon 8	Glu 290 OCH (G-->T)	G/G G/T	91 1	G = 0.995 T = 0.005	1
Intron 8	IVS8 +28 (G-->A)	G/G G/A	91 1	G = 0.995 A = 0.005	1
Intron 9	IVS9 +1319 (C-->G)	C/C C/G	88 1	C = 0.994 G = 0.006	1

Table 7 continued

Sequencing region	SNP	Genotypes	#	Allele frequencies	HWE P value
Intron 10[1]	IVS10 +30 (C insertion)	T/T C insertion	79 10	DEL=0.944 INS=0.056	1
Exon 11	Leu 413 Phe (G-->C)	G/G G/C	87 1	G = 0.994 C = 0.006	1
Exon 13 Genotyping	Gly 633 Asp (G-->A)	G/G G/A A/A	55 24 4	G = 0.807 A = 0.193	0.441
3'UTR	IVS13 +25 (rs3733545) (G-->T)	G/G G/T T/T	46 31 3	G = 0.769 T = 0.231	0.683

[1]. Nucleotides IVS10 +30 ~ IVS10 +37 differed from the published sequence CACATGTA. The observed sequence was TGAGGGATG.

2.6.5 Allelic association analysis between human SLC34A2 SNPs and SLC phenotypic variation

Due to the fact that almost all SNPs identified by the resequencing of 94 humans are rare SNPs (minor allele frequency<10%) except for the SNP in exon 13, we selected some common SNPs from both private and public databases to cover the whole gene. The results of human *SLC34A2* allelic association analysis in RFHS phase II are shown in table 8. SNP rs3775909 is significantly associated with phenotypic variations of SLC in individual association test by both SOLAR and FBAT. The p-value (≈ 0.03) in SOLAR and FBAT (-e) are significant individually, but couldn't pass the multiple testing adjustment by Bonferroni, by which the significance level for a single test is set as 0.0125 ($\alpha = 0.05/4$; Four SNPs). However, haplotype association tests

(table 9) shown strong association ($p<0.005$) between block 2 in human *SLC34A2* and SLC even after the multiple test corrections and marginal significant association with SBP and DBP.

Table 8 Results of human *SLC34A2* allelic association tests in Phase II

SNPs ID	rs#	Location	minor allele frequency	HWE (p_value)	SLC (P_ value) FBAT (-e)	SLC (P_ value) SOLAR	SBP (P_value) FBAT (-e)	DBP (P_value) FBAT (-e)
SLC34A2_1	rs12501856	Intron 1	0.18	0.66	0.58	0.72	0.35	0.3
SLC34A2_2	**rs3775909**	Intron 3	0.34	0.79	**0.03**	**0.03**	0.29	0.3
SLC34A2_3	rs3796777	Intron 9	0.13	0.38	0.98	0.78	0.86	0.81
SLC34A2_4	Gly633Asp by resequencing	Exon13	0.13	0.2	0.13	0.18	0.42	0.48

The significance level for a single test is set as $p=0.0125$ ($\alpha = 0.05/4$; Four SNPs).

Table 9 Summary of HBAT (Haplotype association analysis) results

Haplotypes		Estimates of frequency	FBAT (-e) P value		
			SLC	SBP	DBP
h1	C T A G	0.433	0.23	0.38	0.36
h2	**C C A G**	0.295	**0.002**	**0.08**	**0.09**
h3	G T G G	0.079	0.85	0.61	0.52
h4	G T A G	0.048	0.53	0.2	0.2
h5	C T A A	0.048	0.13	0.49	0.46
h6	G T G A	0.041	0.71	0.78	0.84
h7	**C C A A**	0.037	**0.014**	**0.01**	**0.01**
h8	G T A A	0.011	0.17	0.25	0.29
h9	C T G G	0.007			

The significance level for a single test is set as $p=0.0056$ ($\alpha = 0.05/9$; Nine haplotypes).

2.6.6 SNP association analysis between baboon SLC34A2 SNPs and SLC phenotypic variation

Figure 4 LD pattern of all observed SNPs in sequencing baboon *SLC34A2* by haploview

White: D'<1 and LOD <2; Blue: D'=1 and LOD <2; Shades of pink/red: D'<1 and LOD ≥2; Bright red: D'=1 and LOD ≥2.

Variance component method by SOLAR was used for baboon allelic association analysis. Among all genotyped sites in baboon, SNP Asn136Asn (exon5) show (table 10) strong evidence of association with SLC variations (p=0.0001) even after multiple test adjustment by

Bonferroni, by which the significance level for a single test is set as 0.0175 (α = 0.05/3; three SNPs).The genotypes of this single SNP explained about 5% of total variance in SLC.

Table 10 Summary of results of baboon SLC34A2 allelic association analyses

SNPs	Position	minor allele frequency	HWE P value	SOLAR
Asn136Asn	exon 5	0.48	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).

In order to further understand the genotype effect on the means of trait, we calculated the means of SLC activity by each genotype in the Asn136Asn which was significantly associated with SLC (table 11). Interestingly, means of SLC activity among individuals with minor homozygous genotype (CC) is significantly higher than ones with other two genotypes (P=0.0003), which suggests that CC might increase the risk of high SLC.

Table 11 Means of SLC activity by each genotype of Baboon Asn136Asn

SNPs	Baboon		
	Asn 136 Asn		
Genotypes	CC	CT	TT
Number of Individuals (n)	136	277	155
Average SLC activity	0.286	0.229	0.229
P value	0.00003		

2.6.7 Conditional linkage analysis based on baboon Asn136Asn genotype

In order to determine if Asn136Asn is the genetic variant that accounts for the baboon linkage signal identified by Kammerer et al (2001), multipoint linkage analysis conditional on Asn136Asn genotypes was performed. The peak LOD score was only slightly reduced in conditional linkage models from 11.2 to 10.8. Strong evidence of linkage remained in the model after the effect of Asn136Asn genotypes was removed from the model (Figure 5).

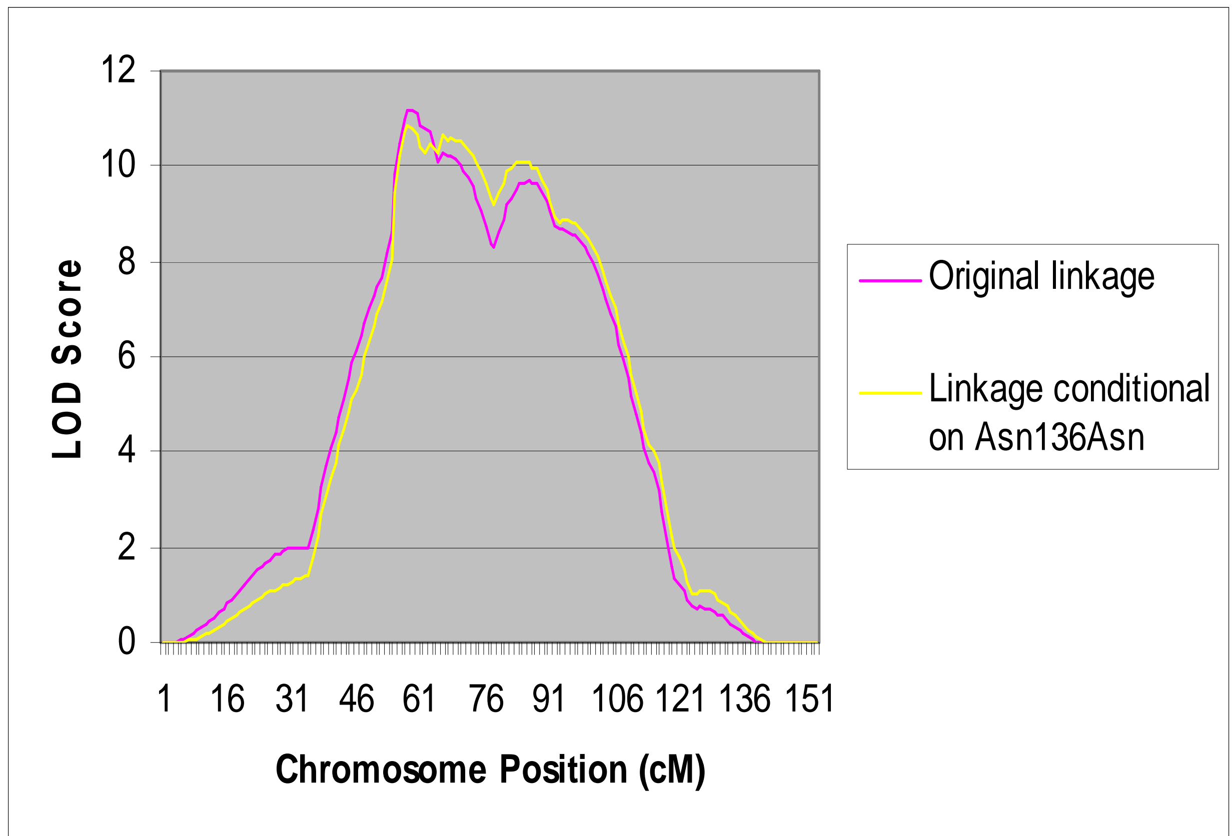

Figure 5 Comparison of the original linkage signals (red line) with the linkage analysis results conditional on Asn136Asn genotypes in baboons on chromosome 5 (homologous to human chromosome 4)

2.7 DISCUSSION

SLC34A2 is located at chromosome 4p15.1-p15.3 (Xu et al. 1999) in a region of the genome homologous to the region of baboon chromosome 5, linked to SLC (Kammerer et al. 2001); therefore *SLC34A2* is a positional candidate gene for SLC. *SLC34A2* was first cloned by Xu et al. (1999) and was reported to be involved in sodium dependent transcellular flux of

phosphate in small intestine (Hilfiker et al, 1998; Xu et al. 1999). The role of *SLC34A2* in other tissues is unclear until recently Corut et al. (2006) reported that mutations in *SLC34A2* cause pulmonary alveolar microlithiasis and might be responsible for calcium deposits in lung and other tissues. We tested the expression of *SLC34A2* in diverse human tissues which might involve in the development of hypertension. It was shown that *SLC34A2* is strongly expressed in lung, kidney and placenta; moderately expressed in arota, left atrium and heart. The active expression of *SLC34A2* in these blood pressure regulation related tissues implied that this gene might play a role in the network of blood pressure control, which made this sodium related solute carrier both a positional and functional candidate gene for SLC.

Resequencing of *SLC34A2* in the baboon and the human establishes the strong homology in exonic organization and sequence between the human and baboon *SLC34A2* genes and extensive variation in both species (see figure 3). Genotyping of SNPs of *SLC34A2* in baboon revealed one variation Asn136Asn on exon 5 is significantly ($p=0.0001$) associated with phenotypic variation in sodium-lithium countertransport. The proportion of the SNP explained variation in SLC is around 5%. In human, the SNP rs3775909 located in intron 3 was significantly associated with SLC ($p=0.03$) with SNP effect less than 1%, but it failed to pass a multiple testing adjustment. However, strong association ($p<0.005$) was observed through haplotype analysis and was still significant even after adjustment for multiple testing. There are several possible explanations for why allelic effect is larger in baboons than in humans.1) the living environment of baboons are more homogeneous than that of humans, for example, baboons were fed the same food and water. 2) Differences between the two species. 3) Specific genotype effects of different SNPs.

To further understand which genotype of these SNPs affected the trait, we compared the means of SLC activity by each genotype of baboon Asn136Asn. It's very interesting that much higher levels of SLC activity were observed in the less common C/C genotype compared to C/T and T/T genotypes, while similar levels between C/T and T/T groups in Asn136Asn. This may suggest that the CC genotype of this SNP may increase the risk of high SLC through a recessive effect.

We also observed that covariates triglycerides (p=1.1e-13), BMI (p=3.2e-7) and sex (p=0.04) are significantly associated with SLC in human. Cholesterol, HDL and LDL are collinear with triglycerides; age is partially collinear with BMI; the approximately explained variance for triglycerides is around 5%. This is consistent with previous studies by Herlitz et al.(2001) and Wierzbicki et al.(2001). We find no association between *SLC34A2* and triglycerides (p=0.61), which suggested that the effect of *SLC34A2* on SLC was not through triglycerides.

To address the question if the SNP Asn136Asn account for the linkage signal identified by Kammerer et al. (2001), conditional linkage analysis which incorporated the measured genotype effect of this SNP into model was conducted. After removing the SNP effect, strong evidence of linkage remained in the conditional analyses. There are several possible explanations for why this polymorphism showed strong association with SLC but only accounted for mild linkage signal. Since the association analysis is far more powerful than linkage analysis in detecting the common variants with modest effects, it's likely that Asn136Asn is only one of several functional variants. There are still other polymorphisms with larger effect in this gene that exist. It is also possible that the linkage signal was explained by several genes, *SLC34A2* being one of them but not the major effect gene. Alternatively, Asn136Asn itself may not be a

functional site or the association is spurious, however the consistent evidence of associations of SLC with *SLC34A2* in two different species makes the last explanation less possible. Because our study design was family-based, including ascertainment of relatively large family-based samples and application of family-based association tests (FBAT), which avoided the effect of major confounding factor-population stratification in association study. Also, the very small p-values of association tests in both species make the likelihood of false positive result very unlikely.

Our study focused on finding functional variants, so we only resequenced the coding regions, splicing regions and predicted promoter regions of *SLC34A2*; also we only genotyped the common sites with functional importance in baboon and some most common SNPs in human. There are certainly additional polymorphisms that need to be tested. We are currently using the Illumina beadarray to genotype tag SNPs at human *SLC34A2* in RFHS phase II population. On the other hand, in this study we primarily investigated the structure variants of *SLC34A2*. To thoroughly assess the role of *SLC34A2* in SLC, systematically assaying the association of *SLC34A2* gene expression levels with SLC is necessary. We are planning to test the expression levels of this gene in individuals with extreme SLC activities by taqman real time PCR.

2.8 CONCLUSION

In conclusion, our study provided strong evidence that *SLC34A2* is significantly associated with phenotypic variation in sodium lithium countertransport in both human and baboon, though with only moderate effect. The explained variance of SLC by single SNP

Asn136Asn in baboon *SLC34A2* is around 5%, and SLC is significantly higher (p=0.0003) in rare homozygous genotype CC of Asn136Asn. However the SNP Asn136Asn in baboon *SLC34A2* might not be the primarily functional site responsible for SLC, there seems to be other variants with larger effect in or near *SLC34A2* accounting for the largest part of the linkage signal in baboon. Further studies need to be done to thoroughly screen this gene and regions next to it.

3.0 SEARCHING FOR CANDIDATE GENES FOR SLC BY COMBINING GENE EXPRESSION PROFILES WITH LINKAGE ANALYSIS

3.1 SPECIFIC AIM

To identify genes for SLC by integrating data from gene expression profiling with linkage data.

3.2 BACKGROUND

3.2.1 Gene expression profiling in hypertension

Gene expression microarray analysis (GEMA), which measures the relative expression levels of tens of thousands genes simultaneously, has shed light in the investigation of hypertension. cDNA and oligonucleotide microarrays are the most commonly used methods now. Although it has been criticized as a non-hypothesis driven, descriptive study with noisy data due to both the experimental and biological variation, GEMA has helped with identifying novel genes whose expression show unexpected relationships to blood pressure and generating new hypotheses to untangle the web of pathways leading to hypertension (Barta et al., 2002; Liang et al., 2002). Besides every effort including sufficient replicates, standardization of the

experimental protocol, and optimization of statistical methods must be made to minimize the noise in GEMA data, extensive work is needed in order to respond to other challenges in the GEMA study. We still don't know which cell types or tissues are most important to study in the pathogenesis of essential hypertension, and it's difficult to determine whether the changes in gene expression level reflect primary genetic mechanisms contributing to hypertension or simply secondary responses to the increased blood pressure. One way to overcome this problem is to combine genetic strategies with gene expression profiling, including applying gene profiling in genetically selected animals, such as congenic strains or transgenic strains (Aitman et al.,1999; Monti et al., 2001; Eaves et al., 2002); and combining gene expression profiling with linkage analysis.

3.2.2 Combining gene expression profiling with linkage analysis to identify candidate genes.

GEMA can generate a long list of differentially expressed genes, but many of them are not disease related genes. Recently, two different integrative approaches for combing genetic linkage with expression profiling have been reported to successfully guide the focus on high-priority genes.

One approach (Yagil et al. 2005) integrated data from expression profiling with genetic linkage data. The assumption is that differentially expressed genes which also map within the linkage region will be the best possible candidate genes.

The other approach (Hubner et al. 2005 and 2006) treated the expression profiling as an expression phenotype, which was used in genetic linkage to generate expression quantitative trait locus (eQTL). The loci which mapped physical phenotype in linkage was called physical

quantitative trait locus (pQTL). The assumption is the eQTL genes that colocalize with pQTL will be the highly likely candidate genes.

The strength of the first approach is that it is straightforward and relatively inexpensive; the strength of the second approach is that it can distinguish 'causative' gene (the alteration of the gene expression is due to the polymorphisms in or near this gene) and 'regulatory' gene (the variants in this gene effect the expression of another gene rather than itself). We plan to use the first approach to investigate the candidate genes for SLC.

3.2.3 Epstein-Barr Virus- transformed human lymphoblastoid cell lines

The use of normal human cells has been restricted by their limited proliferative potential. Epstein-Barr Virus- transformed human lymphoblastoid cell lines are transformed cell lines with the characteristics of continuous proliferation and cellular homology. Therefore, it has been widely used in many types of research and has achieved success in studies of various inherited diseases (Miller 1990; Nilsson 1992, Deutsch et al. 2005).

3.3 EXPERIMENTAL DESIGN

This specific aim will be accomplished through the completion of the following steps:

- Isolating RNA from 12 lymphoblastoid cell lines from individuals with extreme phenotype (High verse Low level of SLC) belonging to RFHS phase II.
- Identifying differentially expressed (DE) genes by duplicate affymetrix oligonucleotide microarrays.

- Searching for DE genes which map within the peak linkage region for SLC in RFHS phase II (Linkage data came from Dr. Alanna Morrison).
- Allelic association analysis between the candidate genes and sodium lithium countertransport phenotypic variation.

3.4 MATERIALS AND METHODS

3.4.1 Cell culture

12 Epstein-Barr virus transformed lymphoblastoid cell lines were established according to a routine method (Henderson et al, 1983; Neitzel 1986) and supplied by Dr. Alanna Morrison. The donor individuals belonging to RFHS phase II with extreme phenotypes (cases were selected from individual with SLC >410 umol/l RBC/hr and controls were selected from individual with SLC <190 umol/l RBC/hr) of sodium lithium countertransport (table 1). All 12 cell lines were quickly thawed at 37°C water bath on the same date. Transfer cells to a 15ml centrifuge tube, bring the volume to 10ml with RPMI 1640 medium (Mediatech, Inc. VA), centrifuge at 1000rpm for 6 minutes. Remove all but 0.5ml of supernatant and resuspend cell pellet in 10ml of RPMI 1640 working media - 85% RPMI 1640 medium 15% FBS (Mediatech, Inc., Herndon, VA), 100 U/ml penicillin, and 100 mg/mL streptomycin (Biowhittaker, Frederick, MD), 2mM L-glutamine (sigma-Aldrich Corp. St. Louis, MO). Transfer cell suspension to T-25 tissue culture flasks and incubate under identical conditions (37°C with 5% CO_2, 21% O_2, 74% N_2). The degree of confluence and the state of bacterial and fungal contamination were monitored by using an inverted phase contrast microscope. The cell density was maintained between 0.5 $X10^6$

and $1X10^6$. The cultures were refed the fresh RPMI 1640 working medium every two to three days when the pH of cultures became acidic (appearing yellow), or passaged when the cells clumped (above $1X10^6$ vial cells/ml). When large clumps were observed ($1\sim2X10^6$ cells/ml), one flask of culture was harvested, the others were frozen for the second time microarrays. Briefly, 10ml of the cell suspension was transferred to a 50ml centrifuge tube, and centrifuge at 1000rpm for 6 minutes at room temperature. Aspirate all but 0.ml of supernant. Resuspend the cell pellet in 1.0ml of 10% DMSO solution (0.1ml DMSO, 0.9ml RPMI 1640 working medium) and transfer to a cryovial. The cryovials are placed into a Nalgene cryogenic freezing container (Cole-Parmer, Vernon Hills, IL) which was filled with isopropyl alcohol and placed at -80°C freezer to achieve a 1°C/min rate of cooling. After 24 hours, the vials were transferred to a cryobox and stored in the liquid nitrogen freezer.

3.4.2 RNA isolation and purification

Cells were harvested when the cultures reached approximately $1\sim2.0 \times 10^6$ viable cells/ml. RNA was isolated with Trizol reagent (GIBCO-BRL). In brief, cells were centrifuged at 1000rpm for 8 min at room temperature, and pellets were lysed by adding 1ml of trizol reagent and repetitive pipetting. 0.2ml chloroform was then added to above tubes. After shaken vigorously, the samples were incubated at room temperature for 2 minutes, and centrifuged at 12,000 RPM for 15 minutes at 4°C. The aqueous phase was transferred into new tubes. 0.5ml isopropanol was added and incubated at room temperature for 10 minutes. After centrifuged at 12,000 RPM for 10 minutes at 4°C, pellets were washed with 1ml 75% ethanol. DNA was dried in air for 10 minutes.

RNA was then purificated by RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. Adjust samples to a volume of 100ul with RNase-free water. Add 350ul buffer RTL and mix thoroughly. Add 250ul ethanol to the diluted RNA, pipetting thoroughly. Transfer samples to an RNeasy mini column placed in a collection tube. Centrifuge at 10,000 rpm for 30sec. Discard the follow-through. Add 500ul buffer RPE onto the RNeasy column, centrifuge at 10,000 rmp for 30 sec. Repeat this step by ending with centrifuge at 10,000 rpm for 2 min. Transfer the RNeasy column to a new collection tube and elute the RNA by adding 45ul RNase free water to the spin column and centrifuge at 10,000 rpm for 2 min.

The concentration of RNA was measured by absorbance at 260nm on a spectrophotometry (GeneQuant, Pharmacia Biotech). All samples have 260/280 nm ratio between 1.8~2.0. Integrity of total RNA was assessed by electrophoresis on a denaturing agarose gel follwing Ambion's (Austin, TX) protocol. Heat 1 g agarose in 72 ml water until dissolved, when cool to approximately 60°C, add 10 ml 10X MOPS running buffer (0.4 M MOPS, pH 7.0, 0.1 M sodium acetate, 0.01 M EDTA), and 18 ml 37% formaldehyde (12.3 M), pour the gel to a tank. Prepare the RNA samples by adding 2 μl of formaldehyde load dye (Ambion Inc., Austin, TX) and 0.8ul ethidium bromide (500ug/ml) into1ul RNA sample. Heat the sample to 65°C for 5 min and immediately place on ice. Load the samples to the gel and electrophorese in 1XMOPS running buffer at 100 Volts for 1.5 hours. Visualize the gel on a UV transilluminator. All 28S and 18S rRNA bands were sharp and clear with a 28s/18s rRNA ratio approximately 2.0. The quality of RNA was double-checked by core lab of University of Pittsburgh using an Agilent 2100 BioAnalyzer with the RNA 6000 Nano LabChip before starting microarray hybridization.

3.4.3 Microarray and data analysis

Oligonucleotide microarray hybridization was done by the core lab of the University of Pittsburgh. Total RNA was reverse transcribed into cDNA by using GeneChip T7-Oligo(dT) Promoter Primer Kit (Affymetrix, Santa Clara,CA,) as described in affymetrix microarray suite user's guide 5.0. In brief, primer hybridization was done by incubating mixture of 2ul of T7-Oligo(dT) primer, 5ul of high quality total DNA and DEPC-treated water at 70°C for 10 min. Put on ice after quick spin. Add 5xfirst-strand cDNA buffer, 0.1M DTT, 10mM dNTP and superscript II RT to above mixture and incubate at 42°C for 1 hour for first strand synthesis. Second-strand cDNA synthesis was conducted by adding 5xsecond-strand cDNA buffer, 10mM dNTP mix, 10U/ul E. coli DNA ligase, 10U/ul E. coli DNA polymerase I and 2U/ul E. coli RNase H to the first-strand synthesis tube, the final volume was adjusted to 150ul by DEPC-treated water. Double stranded cDNA was then cleaned (Qiagen Genechip Samples Cleanup Module) and synthesized into Biotin-labeled cRNA by using Enzo Bioarray Highyield RNA transcript Labeling Kit (Affymetrix). Biotin-labeled cRNA was cleaned up by Qiagen Genechip Samples Cleanup Module and quality checked by Bioanalyzer 2100. The labeled cRNA was fragmented by incubating with fragment reaction buffer supplied with the Genechip sample cleanup module at 94°C for 35 mins. Target fragmented cRNA was eventually hybridized to Affymetrix human U133A oligonucleotide arrays containing over 22000 probe sets. The entire experiment was conducted independently in replicate by using the passages from the first time cultured cell lines under identical conditions; human U133_plus_2.0 oligonucleotide microarrays (Affymetrix, Santa Clara,CA) with over 54000 probe sets were applied.

After washing and staining, the probe arrays were scanned by Affymetrix Genechip Scanner 3000 and scaled by Affymetrix Microarray Suite 5.0. The intensity value of each probe

was saved in .cel files. The array quality was assessed by the percent genes present, hybridization controls: BioB, BioC, BioD, cre as well as internal control gene (GAPDH). The percent genes present was similar across all arrays and approximately 40%~50%. The 3' to 5' ratio of GAPDH was around 1.0 for all arrays. The intensities of the BioB, BioC, BioD and cre probe sets were approximately equal for each array.

In affymetrix oligonucleotide microarrays, each gene is represented by a probe set of 16-20 pairs of probes, which are usually 25-mer oligonucleotides. Each probe pair is composed of a perfect match (PM) and a mismatch (MM) probe, which has same oligonucleotides as PM except for a change at the middle base. In order to use the intensity information of 16-20 probe pairs to represent the gene expression level of a given gene, the probes intensity data in .cel file was log2 transformed and normalized by the Robust Multi-array Average (RMA) algorithm (Irizarry, et al. 2003; Bolstad, et al. 2003) in Bioconductor (Gautier, 2002). The RMA adjusts the probe-level data in three stages, including model-based background correction, quantile normalization and iterative median polishing procedure. A robust linear model which removes probe specific affinities was used in the RMA algorithm.

Data was filtered after RMA normalization; only probes with the average log2 expression value across all samples of more than 5.0 and standard deviation of more than 0.1 were considered.

An empirical Bayesian Method for analyzing one-channel microarray data (Lin et al. 2003) using the statistical package R (R, Version 2.1.0; R Foundation for Statistical Computing, Vienna, Austria) (Becker et al. 1988) was used to rank genes. The ranking of the genes is based on the log odds of differential expression of each gene. This method is a simplification of the empirical Bayes method proposed by Lönnstedt and Speed (2002) extended to unpaired data. It

performs much more reliably than a T-test for a small number of replicates of microarrays. The common genes among the top 220 (approximately 1% of all the probes of U133A) differentially expressed (DE) genes in U133A arrays and top 540 (1% of all probes of U133_plus_2.0) DE genes in U133_plus_2.0 arrays, which also map within the 1 LOD confidence interval of linkage signal for SLC were selected as candidate genes. Genes within the peak linkage regions, but only differentially expressed in U133_plus_2.0 arrays were also considered, due to the fact that the U133_plus_2.0 oligonucleotide arrays comprise a lot of probes that U133A arrays did not include.

The function classifications of the top 540 DE genes in the U133_plus_2.0 arrays were explored by using Ingenuity Pathways Analysis (IPA) (Ingenuity systems, www.ingenuity.com), a web-based knowledgebase and analysis tool. Over-expressed canonical pathways or molecular functions were identified simultaneously by IPA. After assigning these 540 genes into different functions based on the IPA biology database, it compares the numbers of genes appearing at a given function/pathway with their occurrence in all IPA annotated functions/pathway. Only function/pathway annotations containing more genes than expected by chance are considered as over-represented and the significance is expressed as a P value using a right tailed Fisher's exact test.

(Microarray data analysis was carried out in collaboration with Dr. Eleanor Feingold and Dr. Brian Reck).

3.4.4 Genetic linkage analyses for SLC in RFHS all generation

Merlin variance-components method was conducted in linkage analysis in all generations for RFHS Phase II (This part of work was done by Dr. Alanna Morrison's lab). CNT by itself,

log-transformed CNT, and CNT and log-transformed CNT adjusted for age, age-squared and gender were tested. Multipoint IBD (identity by descent) was calculated by using Lander-Green algorithm with sparse gene flow trees. A QTL (Quantitative trait loci) with LOD ≥1.50 was considered as a tentative candidate, with LOD ≥2.00 as suggestive, with LOD ≥3.00 was considered in significant linkage with the trait.

3.4.5 Genotyping

High throughput TDI-FP was used to genotype the candidate genes as described before.

3.4.6 SNP association analysis

SOLAR was used to assess the allelic association of candidate genes with the phenotypic variation in all generations of RFHS phase II. The procedures were same as before.

3.5 RESULTS

3.5.1 Characteristics of subjects

Table 12 summarizes the clinical characteristics of all 12 subjects. The age, sex, BMI, cholesterol, blood pressure, triglycerides were not significantly different between groups. However, the SLC activity level was significantly elevated in High SLC group compared with Low SLC group.

Table 12 Clinical characteristics of all 12 subjects used in microarrays

High SLC group	SEX	AGE (Year)	BMI (kg/m^2)	SBP (mmHg)	CHOL (mg/dl)	TRIG (mg/dl)	SLC activity (umol/l RBC/hr)
H1	F	20.0	21.3	93.3	171.0	75.0	520.4
H2	M	53.0	30.2	141.3	278.0	251.0	492.0
H3	F	63.5	28.7	149.0	245.0	159.0	578.6
H4	F	41.5	23.7	99.0	232.0	88.0	488.7
H5	F	37.7	28.0	98.7	185.0	133.0	418.4
H6	F	45.1	32.1	99.0	164.0	83.0	472.3
Average	1M/4F	43.5	27.3	113.4	212.5	131.5	495.1
standard deviation		14.7	4.1	24.8	46.0	67.0	53.0
Low CNT group							
L1	M	12.8	20.2	94.0	151.0	243.0	169.5
L2	M	77.6	21.7	163.7	192.0	89.0	180.1
L3	F	79.6	31.0	155.0	195.0	131.0	183.1
L4	F	42.3	19.6	89.0	228.0	50.0	136.8
L5	F	46.1	21.8	91.7	183.0	59.0	128.7
L6	F	45.3	28.1	86.0	210.0	97.0	124.3
Average	2M/3F	50.6	23.7	113.2	193.2	111.5	153.8

Table 12 continued

	SEX	AGE	BMI	SBP	CHOL	TRIG	SLC activity
standard deviation		25.0	4.7	35.9	26.0	70.6	26.8
High/Low CNT (t test P value)		0.56	0.19	0.99	0.40	0.63	1.32E-06

3.5.2 Linkage regions by MERLIN

Table 2 lists the suggestive linkage regions discovered by running MERLIN variance-components method. The tentative linkage regions overlapped with those reported earlier (Schork et al. 2002; Hasstedt et al. 2004) were also indicated. It's shown that our linkage results are concordant with the suggestive QTL for SLC of previous study.

Table 13 Suggestive linkage region for SLC by MERLIN

Chromosome	cM	LOD	Cytogenic region	location(cM) of peak marker reported
2	110, 190	2.57, 1.4	2q11.2, 2q31.1	204 (Schork et al. 2002)
3	40, 100, 160	1.46, 3.15, 1.4	3p21.3, 3p11.2, 3q23	45 (Hasstedt et al.2004); 149 (Schork et al. 2002)
6	40, 70, 145	2.38, 2.26, 1.81	6p21.2, 6q11.2, 6q23.1	73 (Schork et al. 2002)

3.5.3 Functional classification of DE genes

Functional classifications of the top 540 differentially expressed genes from array U_133_plus2.0 were done by using Ingenuity Pathway Analysis. There were a total of 492 annotated genes found in Ingenuity IPA database. These were classified into 68 different function/disease groups. The top ten over-represented molecular and cellular functional annotations are shown in Figure 6.

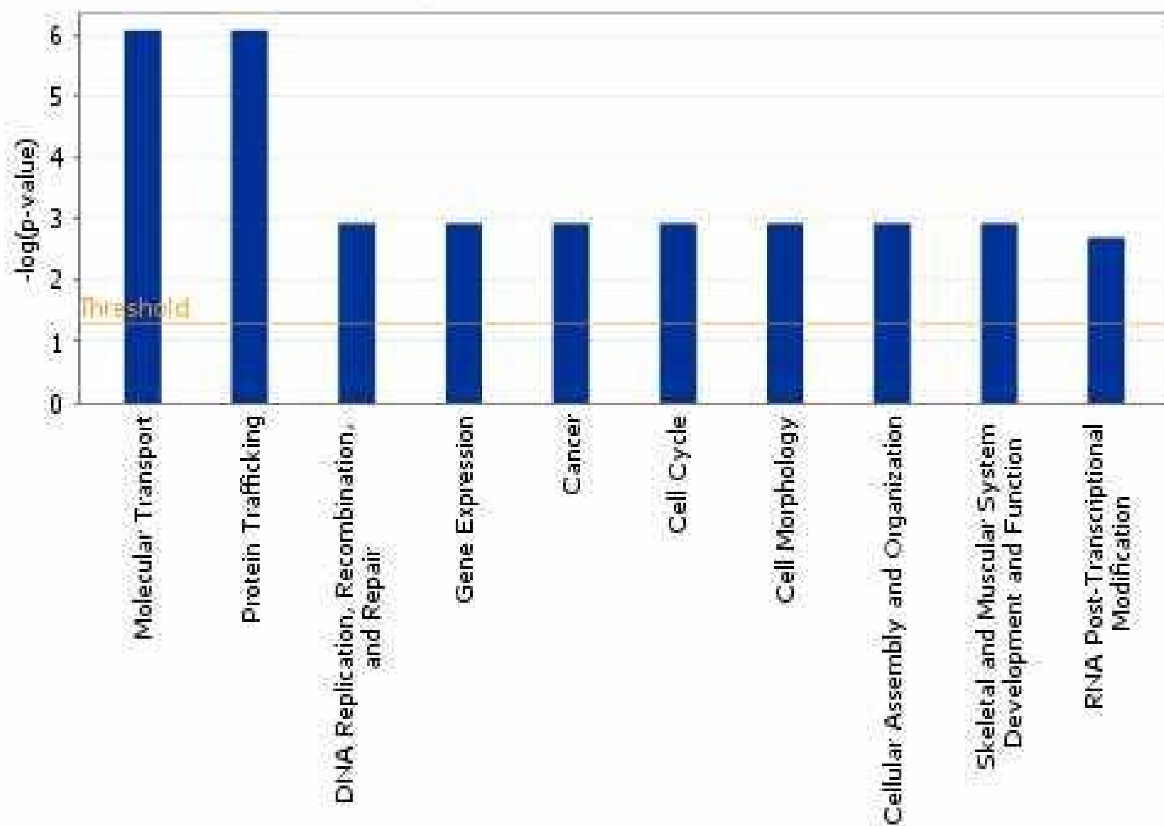

Figure 6 Top 10 over-represented functional annotations from DE genes in array U_133_plus2.0

Figure 6 was generated through the use of Ingenuity Pathway Analysis (Ingenuity systems, www.ingenuity.com). Y-axis shows –log10 transformed P values. The X-axis shows the top 10 over-represented molecular and cellular functional annotations. Among them, molecular transport and protein trafficking are the most significantly over-represented functional annotations. Any –log (P value) lower than indicated threshold means that over-representation may be only due to chance.

3.5.4 Combining gene expression profiling and linkage analysis

After filtering, 12253 probes in the U133A array and 22680 probes in the U133_plus_2.0 array were used for further analysis. Common genes between the top 220 differentially expressed (DE) genes from individuals with high SLC activity as compared to low SLC controls in U_133A and the top 540 DE genes in U133_plus_2.0, which also located within the suggestive linkage region for SLC are listed in table 3. We found five genes were differentially expressed in both arrays and map on suggestive linkage regions on chromosome 2, 3 and 6 respectively. Two out of them, IER3 and PRKRA, were increased in high SLC individuals compared with normal controls, while the other three genes, CD47, ARHGAP15 and CDKAL1, were decreased in high SLC individuals. We further tested the allelic association between IER3 and SLC. The primers and conditions for PCR and TDI-FP are listed in table 14.

Table 14 Common DE genes between two arrays which also map within the linkage regionS for SLC

Gene name	Gene symbol	Cytoband
Rho GTPase activating protein 15	ARHGAP15	2q22.2
protein kinase, interferon-inducible double stranded RNA	PRKRA	2q31.2
CD47 antigen (Rh-related antigen, integrin-associated signal transducer)	CD47	3q13.1-q13.2
immediate early response 3	IER3	6p21.3
CDK5 regulatory subunit associated protein 1-like 1	CDKAL1	6p22.3

DE genes between High SLC and Low SLC individuals identified only in array U133_plus_2.0 and located in linkage regions are shown in table 15. There were 19 genes that were found differentially expressed in U133_plus_2.0 and also located within the SLC linkage regions.

Table 15 DE genes in array U_133 plus 2.0 located at linkage regions for SLC

Gene name	Gene symbol	Cytoband
COBW domain containing 2	CBWD2	2q14.1
aspartyl-tRNA synthetase	DARS	2q21.3
DnaJ (Hsp40) homolog, subfamily C, member 10	DNAJC10	2q32.1
lymphocyte antigen 75	LY75	2q24
integrin, alpha 4	ITGA4	2q31-q32
ankyrin repeat domain 57	ANKRD57	2q13
bridging integrator 1	BIN1	2q14
WAS/WASL interacting protein family, member 1	WASPIP	2q31.1
integrin, alpha 4	ITGA4	2q31.3
chromatin modifying protein 2B	CHMP2B	3p11.2
TATA element modulatory factor 1	TMF1	3P21-P12
ADP-ribosylation factor 4	ARF4	3p21.2-p21.1
methyl-CpG binding domain protein 4	MBD4	3q21-q22
ring finger protein 7	RNF7	3q22-q24
RAN binding protein 9	RANBP9	6p23
solute carrier family 17 (anion/sugar transporter), member 5	SLC17A5	6q14-q15
mannosidase, alpha, class 1A, member 1	MAN1A1	6q22
malic enzyme 1, NADP(+)-dependent, cytosolic	ME1	6q12
heat shock 70kDa protein 1A	HSPA1A	6p21.3

Table 16 Primers and conditions for genotyping IER3

SNPs ID	Primers		Annealing temp	Mg2+ (mM)	Cycle
rs8512	F	GAAGGAGAGCGTCGTTAA	54°C	2.5	35
	R	CACCAGACTTCATCCCAG			
	FP-F	CAAGGGTGTGAGATGTTCCC	55°C		35
	FP-R	AGAGACCTGCATTTACAGCAG			
rs3094124	F	CTGATGGCTGAAGAGGGTG	54°C	1.5	35
	R	CAAGTTGCCTCGGAAGTC			
	FP-F	CGCCCACCCCTGTGTCC	55°C		35
	FP-R	TTAAAGGGCTCGAGGACGG			
rs2284174	F	GTACTATGCTCAGTACCTG	54°C	2.5	35
	R	GTTGCCCAGGCTCCTGAG			
	FP-F	CCTGCACATATACCCCAGAAT	55°C		35
	FP-R	CTCCCTCCCCAACTTTTATTTTA			

IER3 has only two exons, and three published SNPs in HapMap. One SNP has very small minor allele frequency (MAF=0.027%). So we decided to genotype the other two SNPs, rs8512 and rs309412. We also selected another SNP rs2284174 from database SNPper (CHIP Bioinformatics Tools, Riva and Kohane, 2002). It is shown by SOLAR (table 17) that SNP rs2284174 is marginal significantly associated with SLC (p=0.056) and SBP (p=0.085); SNP rs8512 significantly associated with SBP (p= 0.002) and DBP (p=0.0008), but not associated with SLC; SNP rs3094124 is associated with neither SLC nor blood pressure.

Table 17 Association analysis of SLC with IER3 gene by SOLAR

SNP ID	Location	Allele Change	Amino acid change	Minor Allele Freq.	HWE	P value (SOLAR)		
						SLC	SBP	DBP
rs2284174	Promoter	C/T	None	0.21	0.89	0.056	0.085	0.14
rs3094124	Exon 2	C/G	A/P	0.07	0.64	0.73	0.64	0.45
rs8512	Exon 2	A/G	None	0.13	0.91	0.55	0.002	0.0008

3.6 DISCUSSION

We characterized the gene expression profiles of Sodium Lithium Countertransport by using the EBV transformed lymphocytes. Functional analysis by ingenuity Pathway analysis has shown that functional annotations including Molecular Transport, Protein Trafficking, Cellular Assembly and Organization, Gene Expression were most overrepresented in the profilings. These findings suggest genes involved in above pathways may play roles in SLC activity regulation, which is consistent with the hypotheses proposed by Demaurex et al. (1994; Canessa 1994; Zerbini et al.2003; Bianchini and Poussegur 1994) that SLC may be operated by a mechanism involving the cytoskeleton or by way of altering the membrane component or membrane transport.

Linkage analysis by Merlin found several suggestive loci. Loci on chromosome 2, 3 and 6 are extremely interesting, which are overlapped with the linkage and association regions reported before (Schork et al. 2002; Hasstedt et al. 2004). By combining gene expression profiling with linkage analysis, we found five commonly differentially expressed genes, ARHGAP15, CD47, IER3, PRKRA and CDKAL1, that are located within suggestive linkage regions for sodium lithium countertransport. Expression of IER3 and PRKRA were increased in high SLC individuals, while the other three genes, ARHGAP15, CD47 and CDKAL1, had decreased expression.

Immediate early response 3 (IER3) is a stress-inducible gene, which encodes a 153-amino acid protein. It's widely expressed in epithelial and endothelial tissues, especially in the vascular endothelium (Feldmann et al. 2001). Some factors such as mechanical and oxidative stress associated with hypertension may influence the expression of this gene (Hamet et al. 1995; Wernig et al. 2002). Ohki et al (2002) and Keiji et al. (2003) investigated the molecular response of macrophages to mechanical stress, associated with hypertension, by cDNA microarray and found that IER-3 was one of the only three differentially expressed genes which were induced with fold change of more than 2.5. Most recently, Sommer et al (2006) showed strong evidence of association of IER3 with development of hypertension and cardiac hypertrophy. They found that the blood pressure was increased 20-25mmHg in IER3 knockout mice, the potential mechanism of which might involve nitric oxide pathway and peripheral resistance. They proposed that IEX-1 might influence vascular smooth muscle cell tone and intracellular calcium concentration by interactions with calcium-modulating cyclophilin ligand (CAML). Our study found that SNP rs8512 in IER3 was significant association with SBP and borderline association with DBP; another SNP rs3094124 in IER3 was associated with SLC. These findings suggest

IER3 could be a very interesting gene for SLC and/or hypertension. We're searching for SNPs from other database for genotyping in order to see if there's a variant or haplotype in IER3 associated with both SLC and blood pressure.

Rho GTPase activating protein 15 (ARHGAP15) is a member of ARHGAP family which encode Rho/Rac/Cdc42-like GTPase activating proteins. The Rho GTPase, such as Rac1, plays an important role in actin cytoskeletal regulation (Nobes et al, 1995; Hall 1998). It has been suggested (Hassanain et al., 2007) that over-expression of Rac1 in transgenic mice may cause hypertension through the activation of DADPH oxidase. Strong evidence has shown by the study of Seoh et al (2003) that "ARHGAP15 could be a genuine regulator of Rac1 signaling", and down-regulate Rac1. They also observed the cytoskeleton changes like cell contraction and augment of actin stress fibers after altering the expression of ARHGAP15. In our microarray study, the expression levels of ARHGAP15 were much lower in high SLC individuals, which may result in over-activation of Rac1 and eventually lead to high blood pressure through or accompanied by alteration of SLC activity by a mechanism involving cytoskeletal rearrangement.

CD47 molecule (CD47) encodes an integrin-associated transmembrane protein. CD47 is involved in the regulation of many cellular processes including cell-cell adhension, cell migration and platelet spreading (Cooper et al, 1995; Parkos et al,1996; Chung et al,1997; Yu et al,2006). It has recently been validated (Shinohara et al. 2006) in epithelial cells that the role of CD47 in the regulation of cell migration is through reorganization of the actin cytoskeleton. Rac and Cdc42 may participate in some of the CD47 induced cellular processes (Miyashita et al, 2004; Yoshida et al, 2000). Although it's not clear about the phisiology and function of sodium lithium countertransport, and diverse models have been proposed ((Demaurex and Grinstein

1994; Bianchini and Poussegur 1994; West et al, 1998; Vareesangthip et al,1996; Thomas et al, 1995), there is a common point that all of these hypothetical mechanisms eventually involved the cytoskeleton change. Therefore, both ARHGAP15 and CD47 were potential functional candidate genes for SLC.

Gene PRKRA (protein kinase, interferon-inducible double stranded RNA) is located in cytoplasm and intracellular, which has broad function and is involved in multiple process, including skeletal morphogenesis, response to stress, signal transduction, induction of apoptosis, protein amino acid phosphorylation, et al (Gene Ontology; Entrez gene, NCBI). The function of CDKAL1 (CDK5 regulatory subunit associated protein 1-like 1) is not clear, it may play roles in catalytic activity and metal ion binding (Gene Ontology; Entrez gene, NCBI).

We plan to genotype all the candidate genes identified by our study using the Illumia Bead Array or FP. IER3 is the first gene that has been genotyped. We're still in process of genotyping other genes.

There several issues about our study those need to be discussed. It's the advantage of our study that we used cell lines instead of tissue samples, because expression profiles from cell lines are much cleaner than from tissues which contain many heterogeneous cells. In addition, we are not sure which tissue is most important for pathogenesis of hypertension. However there may be concerns about using EBV transformed cells for microarray experiments, since there have been reports (Carter et al, 2002) regarding EBV induced gene expression change. Due to the fact that our experimental lymphocytes from high and low SLC individuals were immortalized and cultured under the same conditions, the modifications of some genes expression by EBV, if they exist, should be equal in both case and control groups.

There are some limitations of our experiments. Since our study was based on an assumption that the genes should be expressed in the lymphoblastoid cell lines in order to be identified by microarray as candidate genes for SLC, some genes with low or non-expression in lymphoblastoid cell lines could have been missed in our experiment. Also our study has little power to detect a disease-causing gene if it only has structural or functional alteration instead of change at the mRNA level. The relatively small sample size in our study also restricted our power to find the disease causing genes.

We're now planning to conduct another microarray study with larger independent well matched samples to validate our results and find new disease susceptibility genes. For the genes most significantly associated with SLC and hypertension, functional tests on the protein level will be conducted, such as Western Blot.

3.7 CONCLUSION

In conclusion, our study succeeded in exploring some interesting candidate genes for SLC by combining the gene expression profiling and linkage analysis. All five genes that were identified as common top 1% differentially expressed genes in both arrays and mapped in a linkage region, IER3, PRKRA, ARHGAP15, CD47 and CDKAL1, are good functional candidate genes. Among them, expression of IER3 and PRKRA were increased in high SLC individuals, while all other three genes ARHGAP15, CD47 and CDKAL1 showed decreased expression. Follow-up study on IER3 showed that one SNP in IER3 is associated with SBP and DBP, and another SNP is borderline associated with both SLC and DBP. Further studies are needed to be

done to thoroughly investigate the relationships between SLC and IRE3 as well as other candidate genes identified by our experiments.

4.0 SUMMARY

Two studies were designed to search for genetic determinants for sodium lithium countertransport, an intermediate phenotype for essential hypertension.

The first study explored the relationship between a positional candidate gene – *SLC34A2* and SLC. Human *SLC34A2* is located at chromosome 4p15.1-p15.3 in a region of the genome homologous to the region of baboon chromosome 5, linked to sodium lithium countertransport (Kammerer et al. 2001).

We examined the relative expression of *SLC34A2* in diverse human tissues potentially related with blood pressure regulation and found that this gene has relatively high expression in lung, kidney and placenta, moderate in aorta, left atrium and heart, suggesting that *SLC34A2* is functionally potential to be involved in hypertension. Sequencing analysis of this gene showed a strong homology in exonic organization and sequence between the human and baboon *SLC34A2* genes and extensive variation in both species. The informative SNPs in *SLC34A2* were genotyped in 634 members of chromosome 5 linked baboon pedigree and 1856 RFHS Phase II samples. Strong evidence of associations of phenotypic variation of SLC with baboon SNP Asn136Asn (P=0.0001) and human SNP rs3775909 (P=0.03) as well as human haplotype 2 (P< 0.005) were observed, implied that *SLC34A2* may be one of the genes involved in SLC. However, evidence of linkage remained when the linkage analyses conditional on genotypes of baboon Asn136Asn, suggested that Asn136Asn is not the primarily functional site for baboon

SLC QTL. There might be other variants with larger effect in or near SLC34A2 accounting for the linkage signal in baboon.

In second study, we searched for susceptible genes for SLC by combing gene expression microarray data and linkage analysis data for SLC.

Linkage analysis was conducted by using Merlin variance component method in all generations from RFHS phase II. 12 EBV-transformed lymphocytes with two extremes SLC distribution selected from RFHS phase II were used for microarray study. Two independent microarrays (U133A and U133_plus_2.0) were used to identify the differentially expressed genes in "high" vs. "low" SLC groups. Functional analysis by Ingenuity Pathway Analysis showed that functional annotations as molecular transport, protein trafficking and cellular assembly and organization were significantly over-represented in top 1% differentially expressed genes for array U_133_plus 2.0, suggesting that genes might influence SLC by a mechanism of involving these functional pathways. Five genes: ARHGAP15, CD47, CDKAL1, IER3 and PRKRA were successfully identified as differentially expressed in both arrays and mapped within the linkage regions for SLC. Genotype association analysis of IER3 showed that one of the SNP (rs8512) is significantly associated with SBP (p=0.002) and DBP (p=0.0008); another SNP (rs2284174) is marginal significantly associated with SLC (p=0.055) and SBP (p=0.085). We're searching for additional SNPs in IER3 to thoroughly investigate the relationship of IER3 and SLC. Studies on exploring the relationship of SLC with other candidate genes identified by this study are undergoing.

Due to the very low expression of SLC34A2 in EBV-transformed lymphocytes in microarray, we can not make any conclusion on whether the positional candidate gene-SLC34A2 in our first study is differentially expressed in "high" verse "low" SLC groups.

APPENDIX A Comparisons of the nucleotide and amino acid sequence alignments of *SLC34A2*

```
 Human: ..........................................M   1
Baboon: ..........................................-
 Human: ... GGCCGCCGCCTCCAGCCCAGCACCTGCGGAGGGAGCGCTGACCATG 46
Baboon: ... ----------------------------------------------
         •                          *               •

Human:  A  P  W  P  E  L  G  D  A  Q  P  N  P  D  K  Y  L 18
Baboon: -  -  -  -  -  -  -  -  -  -  -  -  -  S  -  -  -
 Human: GCTCCCTGGCCTGAATTGGGAGATGCCCAGCCCAACCCCGATAAGTACCT 96
Baboon: ---------------------------------------AG---------

Human:   E  G  A  A  G  Q  Q  P  T  A  P  D  K  S  K  E    34
Baboon:  -  -  -  T  S  -  -  -  I  T  -  -  -  -  -  -
 Human: CGAAGGGGCCGCAGGTCAGCAGCCCACTGCCCCTGATAAAAGCAAAGAGA 146
Baboon: ---------TA--A-------A----TCA---------------------

 Human: T  N  K  T  D  N  T  E  A  P  V  T  K  I  E  L  L  51
Baboon: -  -  -  -  -  -  -  -  V  -  -  -  -  F  -  -  -
 Human: CCAACAAAACAGATAACACTGAGGCACCTGTAACCAAGATTGAACTTCTG 196
Baboon: ------------------------T------------T----------
```

```
                  •

Human:    P  S  Y  S  T  A  T  L  I  D  E  P  T  E  V  D  D  68
Baboon:   -  T  -  -  -  -  -  -  -  E  -  -  -  -  -  -  -
Human:   CCGTCCTACTCCACGGCTACACTGATAGATGAGCCCACTGAGGTGGATGA 246
Baboon:  ---A-------------------------G-----------------C--
                                      *

Human:     P  W  N  L  P  T  L  Q  D  S  G  I  K  W  S  E    84
Baboon:    -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
Human:   CCCCTGGAACCTACCCACTCTTCAGGACTCGGGGATCAAGTGGTCAGAGA 296
Baboon:  -----------------G--------------------------------
                                                  •

Human:  R  D  T  K  G  K  I  L  C  F  F  Q  G  I  G  R  L  101
Baboon: -  -  -  -  -  -  -  -  -  V  -  -  -  -  -  -  -
Human:   GAGACACCAAAGGGAAGATTCTCTGTTTCTTCCAAGGGATTGGGAGATTG 346
Baboon:  -------------------------G------------------------

Human:    I  L  L  L  G  F  L  Y  F  F  V  C  S  L  D  I  L  117
Baboon:   -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  V  -
Human:   ATTTTACTTCTCGGATTTCTCTACTTTTTCGTGTGCTCCCTGGATATTCT 396
Baboon:  -----------T-----------------T---------------G----
                                        *

Human:     S  S  A  F  Q  L  V  G  G  K  M  A  G  Q  F  F    133
Baboon:    -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
Human:   TAGTAGCGCCTTCCAGCTGGTTGGAGGAAAAATGGCAGGACAGTTCTTCA 446
```

```
Baboon: --------------------------------------------------
                          •

 Human: S  N  S  S  I  M  S  N  P  L  L  G  L  V  I  G  V   150
Baboon: -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
        *
 Human: GCAACAGCTCTATTATGTCCAACCCTTTGTTGGGGCTGGTGATCGGGGTG 496
Baboon: ----T---------------------------------------------
            *

 Human:  L  V  T  V  L  V  Q  S  S  S  T  S  T  S  I  V   166
Baboon:  -  -  -  -  F  -  -  -  -  -  -  -  -  -  -  -
 Human: CTGGTGACCGTCTTGGTGCAGAGCTCCAGCACCTCAACGTCCATCGTTG 546
Baboon: --------------C----------------------------------

 Human: V  S  M  V  S  S  S  L  L  T  V  R  A  A  I  P  I   183
Baboon: -  -  -  -  A  -  -  -  -  -  -  -  -  -  -  -  -
                                                 *
 Human: TCAGCATGGTGTCCTCTTCATTGCTCACTGTTCGGGCTGCCATCCCCATT 596
Baboon: -----------G--------------------------------------
                           •

 Human:  I  M  G  A  N  I  G  T  S  I  T  N  T  I  V  A  L   200
Baboon:  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
 Human: ATCATGGGGGCCAACATTGGAACGTCAATCACCAACACTATTGTTGCGCT  646
Baboon: -----------------------------------------------A--
```

```
Human:    M  Q  V  G  D  R  S  E  F  R  R  A  F  A  G  A    216

Baboon:   -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

Human:  CATGCAGGTGGGAGATCGGAGTGAGTTCAGAAGAGCTTTTGCAGGAGCCA  696

Baboon: ---------------C--A-------------------------------
                                         •



Human:  T  V  H  D  F  F  N  W  L  S  V  L  V  L  L  P  V  233

Baboon: -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

Human:  CTGTCCATGACTTCTTCAACTGGCTGTCCGTGTTGGTGCTCTTGCCCGTG  746

Baboon: ----------------------------------------G-----T---
                               *



Human:   E  V  A  T  H  Y  L  E  I  I  T  Q  L  I  V  E  S 250

Baboon:  -  -  -  -  -  -  -  -  -  V  -  -  -  -  -  -  -

Human:  GAGGTGGCCACCCATTACCTCGAGATCATAACCCAGCTTATAGTGGAGAG  796

Baboon: --------------------T-----TG----------------------



Human:    F  H  F  K  N  G  E  D  A  P  D  L  L  K  V  I    266

Baboon:   -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

Human:  CTTCCACTTCAAGAATGGAGAAGATGCCCCAGATCTTCTGAAAGTCATCA  846

Baboon: ---------------------------A-----------C----------



Human:  T  K  P  F  T  K  L  I  V  Q  L  D  K  K  V  I  S  283

Baboon: -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

Human:  CTAAGCCCTTCACAAAGCTCATTGTCCAGCTGGATAAAAAAGTTATCAGC  896

Baboon: --------------------------------------------------
                                     •
```

```
Human:   Q  I  A  M  N  D  E  K  A  K  N  K  S  L  V  K  I   300
Baboon:  -  -  -  -  -  -  -  T  -  -  -  -  -  -  -  -  -
                          *
Human:  CAAATTGCAATGAACGATGAAAAAGCGAAAAACAAGAGTCTTGTCAAGAT  946
Baboon: ----------------------CC--------------------------


Human:    W  C  K  T  F  T  N  K  T  Q  I  N  V  T  V  P    316
Baboon:   -  -  -  -  -  -  -  M  -  -  M  -  -  -  -  -
Human:  TTGGTGCAAAACTTTTACCAACAAGACCCAGATTAACGTCACTGTTCCCT  996
Baboon: -----------------------T---------G----------------
                                 •

Human:  S  T  A  N  C  T  S  P  S  L  C  W  T  D  G  I  Q   333
Baboon: -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
Human:  CGACTGCTAACTGCACCTCCCCTTCCCTCTGTTGGACGGATGGCATCCAA  1046
Baboon: -A--G---------------------------------------------


Human:   N  W  T  M  K  N  V  T  Y  K  E  N  I  A  K  C  Q   350
Baboon:  T  -  -  I  -  -  -  -  -  -  -  -  -  -  -  -  -
Human:  AACTGGACCATGAAGAATGTGACCTACAAGGAGAACATCGCCAAATGCCA  1096
Baboon: -C---------A--------------------------------------
                        *                       •

Human:    H  I  F  V  N  F  H  L  P  D  L  A  V  G  T  I    366
Baboon:   -  -  -  -  -  -  -  -  -  -  -  -  -  -  I  -
Human:  GCATATCTTTGTGAATTTCCACCTCCCGGATCTTGCTGTGGGCACCATCT  1146
Baboon: ---------C--------------------------------T-T-----
                       *
```

```
 Human: L   L   I   L   S   L   L   V   L   C   G   C   L   I   M   I   V    383
Baboon: -   -   -   I   -   -   -   -   -   -   -   -   -   -   -   -   -
 Human: TGCTCATACTCTCCCTGCTGGTCCTCTGTGGTTGCCTGATCATGATTGTC  1196
Baboon: --------A-----------------------------------------


 Human:  K   I   L   G   S   V   L   K   G   Q   V   A   T   V   I   K   K   400
Baboon:  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
 Human: AAGATCCTGGGCTCTGTGCTCAAGGGGCAGGTCGCCACTGTCATCAAGAA  1246
Baboon: --------------------------------------------------


 Human:   T   I   N   T   D   F   P   F   P   F   A   W   L   T   G   Y    416
Baboon:   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
                                               *
 Human: GACCATCAACACTGATTTCCCCTTTCCCTTTGCATGGTTGACTGGCTACC  1296
Baboon: ---------------------------------G----------------
                      •


 Human: L   A   I   L   V   G   A   G   M   T   F   I   V   Q   S   S   S    433
Baboon: -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
 Human: TGGCCATCCTCGTCGGGGCAGGCATGACCTTCATCGTACAGAGCAGCTCT  1346
Baboon: -------------------------------------G------------


 Human:  V   F   T   S   A   L   T   P   L   I   G   I   G   V   I   T   I   450
Baboon:  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
 Human: GTGTTCACGTCGGCCTTGACCCCCCTGATTGGAATCGGCGTGATAACCAT  1396
```

Baboon: --------------------T-----------------A-----------

•

Human: E R A Y P L T L G S N I G T T T 466

Baboon: - - - - - - - - - - - - - - - -

Human: TGAGAGGGCTTATCCACTCACGCTGGGCTCCAACATCGGCACCACCACCA 1466

Baboon: --

Human: T A I L A A L A S P G N A L R S S 483

Baboon: - - - - - - - - - - - - T - - - -

Human: CCGCCATCCTGGCCGCCTTAGCCAGCCCTGGCAATGCATTGAGGAGTTCA 1496

Baboon: -----------------------------------A--------------

Human: L Q I A L C H F F F N I S G I L L 500

Baboon: - - - - - - - - - - - - - - - - -

Human: CTCCAGATCGCCCTGTGCCACTTTTTCTTCAACATCTCCGGCATCTTGCT 1546

Baboon: ---A--

•

Human: W Y P I P F T R L P I R M A K G L 517

Baboon: - - - - - - - - - - - - - - - - -

Human: GTGGTACCCGATCCCGTTCACTCGCCTGCCCATCCGCATGGCCAAGGGGCT 1596

Baboon: ------------------------T--------------------------

Human: G N I S A K Y R W F A V F Y L I I 534

Baboon: - - - - - - - - - - - - - - - - -

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 Human: GGGCAACATCTCTGCCAAGTATCGCTGGTTCGCCGTCTTCTACCTGATCAT  1646
Baboon: --------------------------------------------------

 Human:   F  F  F  L  I  P  L  T  V  F  G  L  S  L  A  G  W  551
Baboon:   -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
 Human: CTTCTTCTTCCTGATCCCGCTGACGGTGTTTGGCCTCTCGCTGGCCGGCTG  1696
Baboon: ---------------------------------------T----------

 Human:   R  V  L  V  G  V  G  V  P  V  V  F  I  I  I  L  V  568
Baboon:   P  -  -  -  A  -  -  -  -  -  -  -  -  -  -  -  -
 Human: GCGGGTGCTGGTTGGTGTCGGGGTTCCCGTCGTCTTCATCATCATCCTGGT  1746
Baboon: --C---------G—C-----------------------------------
                                   *

 Human:   L  C  L  R  L  L  Q  S  R  C  P  R  V  L  P  K  K  585
Baboon:   -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
 Human: ACTGTGCCTCCGACTCCTGCAGTCTCGCTGCCCACGCGTCCTGCCGAAGAA  1796
Baboon: -----------------------C-----T-------------C-----
        *                                           *

 Human:   L  Q  N  W  N  F  L  P  L  W  M  R  S  L  K  P  W  602
Baboon:   -  -  -  -  -  -  -  -  -  -  -  H  -  -  -  -  -
 Human: ACTCCAGAACTGGAACTTCCTGCCGCTGTGGATGCGCTCGCTGAAGCCCTG  1846
Baboon: -----------------------A----------A------A--------
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Human:    D  A  V  V  S  K  F  T  G  C  F  Q  M  R  C  C  C  619
Baboon:   -  -  -  I  -  -  -  -  -  -  -  -  -  P  -  -  -
Human:  GGATGCCGTCGTCTCCAAGTTCACCGGCTGCTTCCAGATGCGCTGCTGCTG 1896
Baboon: ---C------A-------------------T----------C---------

Human:    C  C  R  V  C  C  R  A  C  C  L  L  C  G  C  P  K  636
Baboon:   -  -  -  -  -  -  -  V  -  -  -  -  -  -  -  -  -
                                                 *
Human:  CTGCTGCCGCGTGTGCTGCCGCGCGTGCTGCTTGCTGTGTGGCTGCCCCAA 1946
Baboon: -----------------------T-------C-------------------
                                       * *

Human:    C  C  R  C  S  K  C  C  E  D  L  E  E  A  Q  E  G  653
Baboon:   -  -  -  -  -  -  -  -  K  -  -  -  -  G  -  -  A
Human:  GTGCTGCCGCTGCAGCAAGTGCTGCGAGGACTTGGAGGAGGCGCAGGAGGG 1997
Baboon: -------------------------A---------------G--------C
        *                        *             *

Human:    Q  D  V  P  V  K  A  P  E  T  F  D  N  I  T  I  S  670
Baboon:   -  G  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
Human:  GCAGGATGTCCCTGTCAAGGCTCCTGAGACCTTTGATAACATAACCATTAG 2048
Baboon: -----G---------------C-----------------------------

Human:    R  E  A  Q  G  E  V  P  A  S  D  S  K  T  E  C  T  687
Baboon:   -  -  -  -  -  -  -  R  -  P  -  -  -  -  -  -  -
Human:  CAGAGAGGCTCAGGGTGAGGTCCCTGCCTCGGACTCAAAGACCGAATGCAC 2099
Baboon: -----------------------G----C-A--------------------
                                *   *
```

```
 Human:    A   L                                                    689
Baboon:    -   -
 Human: GGCCTTG ...                                                 2106
Baboon: A------ ...
```

* Polymorphism site

▲ Initial base of each exon

- Same amino acid or nuclear acid base in baboon as in human

Highlighted sequences are functional domains predicted by Bioinformatic Harvester.

Yellow sequences represent Alignment of Na_Pi_cotransport domains (AA/108-269) (AA/381-540)

Green sequences represent Low compositional complexity domain (AA/361-379) (AA/616/644)

Red Sequences represent Transmembrane segment domain (AA/553-575)

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