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Hartnup disorder is caused by mutations in the gene encoding the neutral amino acid transporter *SLC6A19*

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Hartnup disorder (OMIM 234500) is an autosomal recessive abnormality of renal and gastrointestinal neutral amino acid transport noted for its clinical variability. We localized a gene causing Hartnup disorder to chromosome 5p15.33 and cloned a new gene, *SLC6A19*, in this region. *SLC6A19* is a sodium-dependent and chloride-independent neutral amino acid transporter, expressed predominately in kidney and intestine, with properties of system B^0. We identified six mutations in *SLC6A19* that cosegregated with disease in the predicted recessive manner, with most affected individuals being compound heterozygotes. The disease-causing mutations that we tested reduced neutral amino acid transport function *in vitro*. Population frequencies for the most common mutated *SLC6A19* alleles are 0.007 for 517G→A and 0.001 for 718C→T. Our findings indicate that *SLC6A19* is the long-sought gene that is mutated in Hartnup disorder; its identification provides the opportunity to examine the inconsistent multisystemic features of this disorder.

First described in 1956 (ref. 1), Hartnup disorder is characterized by increases in the urinary and intestinal excretion of neutral amino acids[2]. Individuals with typical Hartnup aminoaciduria may be

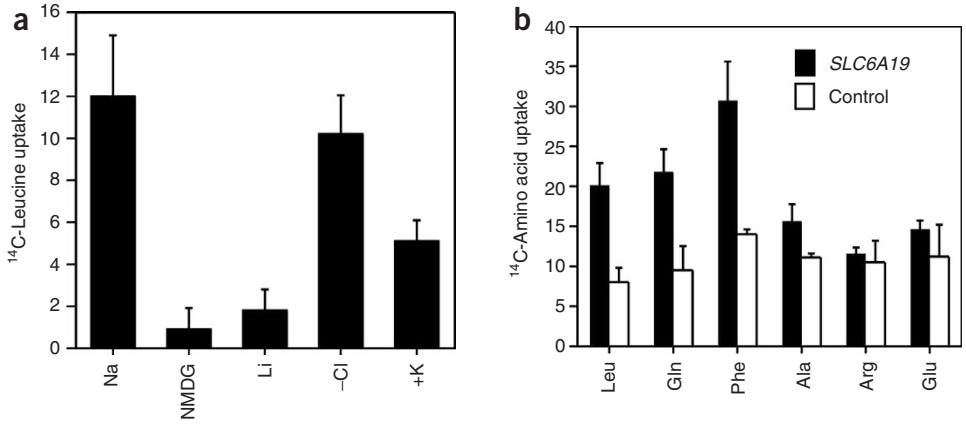

Figure 1 Ion and voltage dependence and substrate specificity of SLC6A19 confirm that it has the predicted profile for system B^0. (**a**) Leucine uptake (pmol per 15 min) in oocytes transfected with *SLC6A19* cRNA in which buffer containing NaCl (Na) was modified. The transport activity of oocytes injected with water was subtracted ($n=3$ experiments). Expression of *SLC6A19* resulted in uptake of 100 μM ^{14}C-leucine at 20±3 pmol per 15 min, which was more than twice that detected in oocytes injected with water (net activity 8±1.8 pmol per 15 min). Uptake of ^{14}C-leucine was sodium-dependent, as replacement of sodium ions by the impermeant N-methyl-D-glucamine (NMDG) or LiCl (Li) reduced the transport activity by 92% or 85%, respectively. Leucine uptake was not chloride-dependent, as replacement of NaCl by sodium gluconate (–Cl) did not significantly alter transport activity ($P>0.1$). Transport of amino acids by SLC6A19 was driven by membrane potential, as the addition of 50 mM KCl (+K) to the transport buffer reduced leucine uptake by 58%. (**b**) Uptake (pmol per 15 min) of ^{14}C-labeled amino acids in oocytes transfected with *SLC6A19* cRNA or controls injected with water. The specificity for neutral amino acids is shown in this experiment, which was done three times. Phenylalanine seemed to be the best substrate, followed by leucine, glutamine and alanine. Glutamate and arginine were not actively transported.

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Table 1 Mutations in *SLC6A19* associated with Hartnup disorder

Mutation	Exon affected	Wild-type sequence	Mutated sequence	Restriction site altered[a]	Affected individuals	Population allele frequency
D173N	4	GAC	AAC	*Xmn*I (c)	2.3, 2.4, 4.4[b], 5.1, 6.3, 7.3	0.007
R240X	5	CGA	TGA	*Dde*I (c)	5.1, 7.3	0.001
L242P	5	CTG	CCG	*Stu*I (d)	1.2, 1.4, 1.5	ND
IVS8+2G[c]	Intron 8 sd	AGgt	AGgg	NA	1.2, 1.4, 1.5	ND
E501K	10	GAG	AAG	*Bpl*I (d)	2.3, 2.4	ND
IVS11+1A	Intron 11 sd	ACgt	ACat	*Bsa*AI (d)	3.4	ND

[a]Restriction site indicated is created (c) or destroyed (d) by the mutation. [b]This individual is homozygous with respect to the allele indicated. All other affected individuals carry one copy of the allele indicated. [c]The IVS8+2G mutation is also described by Kleta *et al.* in this issue[28].
These mutations cosegregated with Hartnup disorder in seven pedigrees. The population frequency of IVS8+2G was estimated by sequencing 50 healthy controls. sd, splice donor site mutations; NA, none available; ND, not detected.

asymptomatic, although some develop a photosensitive pellagra-like rash, attacks of cerebellar ataxia and other neurological or psychiatric features[3]. Although the definition of Hartnup 'disease' was originally based on clinical and biochemical abnormalities, its marked clinical heterogeneity has led to it being known as a disorder with a consistent pathognomonic neutral hyperaminoaciduria. A defective system B^0 neutral amino acid transporter defined by functional studies in brush border membrane vesicles[4,5], bovine epithelial cells[6] and Caco-2 cells[7] is the most commonly named candidate for underlying the Hartnup disorder biochemical phenotype. System B^0 refers to broad amino acids with a neutral (^{0}) charge[8] and is the main transporter for neutral amino acid reabsorption across the luminal membrane of kidney proximal tubules. We previously reported six unrelated asymptomatic Australian pedigrees with the typical biochemical pattern of Hartnup aminoaciduria[9]. In that study, we did not detect causative mutations in a gene encoding a neutral amino acid transporter (*SLC1A5*) on chromosome 19, which was previously suggested to be a candidate for underlying Hartnup disorder[10,11].

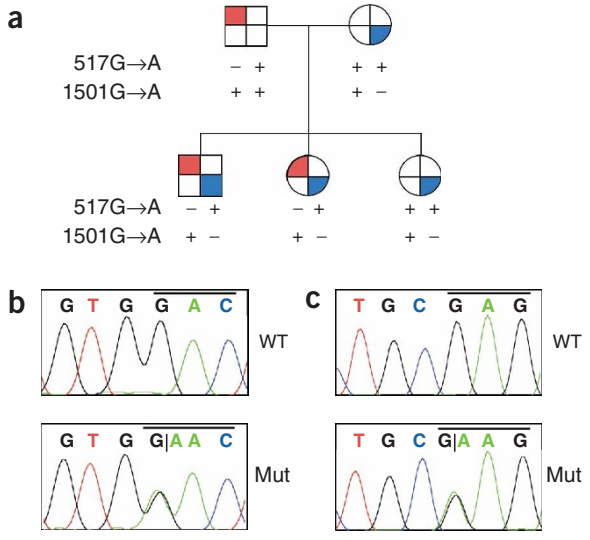

Figure 2 Inheritance of two different mutations of *SLC6A19* in a single pedigree (pedigree 2) in which the son (2.3) and first daughter (2.4) have the Hartnup disorder hyperaminoaciduria phenotype. The second daughter (2.5) and the parents (2.1 and 2.2) are unaffected. (**a**) White quadrants indicate wild-type alleles (+); red and blue quadrants indicate the mutated alleles (–) 517A and 1501A, respectively. The two affected individuals are compound heterozygotes with respect to *SLC6A19*. (**b**) Sequence of the wild-type (WT) and 517G→A (Mut) alleles in the pedigree. (**c**) Sequence of the wild-type (WT) and 1501G→A (Mut) alleles in the pedigree. The affected codon is indicated by a bar over the sequence.

To refine the locus associated with Hartnup disorder, the Australian Hartnup Consortium combined a candidate approach with fine-scale linkage analysis in pedigrees with Hartnup disorder[9] (**Supplementary Fig. 1** online). Five loci were examined for evidence of linkage, owing to their established role in neutral amino acid transport: (i) *SLC3A2* (also called the 4F2 heavy chain)[12–14]; (ii) *SLC7A8* (also called the LAT2 light chain)[12–14]; (iii) *SLC1A5* (also called *ASCT2*; refs. 11,15); (iv) *SLC6A18* (also called *Xtrp2*), an orphan member of the SLC6 family expressed in kidney and intestine[16], and *SLC6A19*, located adjacent to *SLC6A18*, which encodes the human ortholog of mouse B^0AT1 (we identified the latter through homology to *SLC6A19*; ref. 17); and (v) *SLC6A20* (also called *XT3*), a second orphan member of the SLC6 family. These five regions containing candidate genes, their chromosomal locations, their tissue expression profiles and the microsatellite markers studied are shown in **Supplementary Table 1** online. Linkage analyses of the locus associated with Hartnup disorder confirmed the chromosome 5 localization[18] and reduced the candidate region to 3.4 Mb. We obtained maximal evidence for linkage between *D5S392* and *D5S417*, with a maximum lod score of 2.68. No other region examined showed positive evidence for linkage, excluding all other candidates. We rejected one of the two candidates at the refined locus, *SLC6A18* (also called *Xtrp2*)[19,20], because it did not increase neutral amino acid uptake above background levels in *Xenopus laevis* oocytes (data not shown), and because we did not detect mutations in *SLC6A18* that cosegregated with Hartnup disorder.

Subsequent candidate gene analyses focused on *SLC6A19*, the human XTR2-related gene. We cloned the new full-length *SLC6A19* cDNA and found that the mRNA contains a single open reading frame consisting of 1,902 bp encoding 634 amino acids. This information corrected the existing predicted cDNA. Expression of *SLC6A19* in *X. laevis* oocytes showed that it transports neutral amino acids in a sodium-dependent but chloride-independent manner that is driven by membrane potential (**Fig. 1**). Therefore, SLC6A19 has the characteristics of amino acid transport system B^0, the main apical neutral amino acid transporter of kidney and intestinal epithelial cells. These functional analyses are consistent with our recent characterization of the mouse ortholog of *SLC6A19* (ref. 17).

We sequenced the coding region from genomic DNA samples to determine whether mutations in *SLC6A19* could explain the autosomal recessive inheritance and phenotypic variation in pedigrees with Hartnup disorder. We detected six mutations in *SLC6A19* that cosegregated with disease in a simple mendelian manner (**Table 1**). Three mutations (517G→A, resulting in the amino acid substitution D173N; 725T→C, resulting in L242P; and 1501G→A, resulting in E501K) were missense alleles whereas one (718C→T, resulting in R240X) was a nonsense allele. Two mutations (IVS8+1A and IVS11+2G) are predicted to cause aberrant splicing as a consequence

of changes in the donor consensus sequence at exon-intron boundaries for introns 8 and 11, respectively. Only individuals in pedigree 4 are true homozygotes with respect to the D173N allele, whereas all other affected individuals in the other six pedigrees are compound heterozygotes carrying two different mutated alleles. The affected individuals in pedigree 2 are compound heterozygotes with respect to the 517A and 1501A alleles (**Fig. 2**). In addition, we identified two nonsynonymous changes in exon 5, 719G→A (resulting in R240Q) and 754G→A (resulting in V252I), that did not segregate in mendelian fashion with disease or have impaired function (**Fig. 3**). We also detected two synonymous changes, in exon 2 (GGT/GGC) and exon 7 (ACA/ACG), and used them for haplotype assignment. These data indicate that mutations in the coding region and splice sites of *SLC6A19* detected in Australian pedigrees cosegregated with Hartnup disease in the predicted mendelian manner and could account for the biochemical phenotype without further genetic explanation.

We analyzed DNA samples from 580 healthy volunteers to determine whether the observed mutations in *SLC6A19* were present in the unaffected population of European descent. The 517G→A mutation occurred at a frequency of 0.007, suggesting that under Hardy-Weinberg equilibrium, homozygotes should occur at a rate of ~1 in 20,000. This predicted rate of 517A homozygosity is in agreement with previously observed rates for Hartnup disorder in European populations[3,21]. We detected the 517A allele in six parents in five unrelated Hartnup pedigrees (**Table 1**). We detected the 718T allele in the unaffected population and also in two unrelated pedigrees. The other four mutations were not found in the unaffected population. Thus, the frequency of the *SLC6A19* alleles associated with Hartnup disease in the seven pedigrees studied reflects that predicted by the allele frequencies observed in the unaffected population.

We engineered *SLC6A19* to incorporate the mutations found in individuals with Hartnup disorder and introduced the cRNA into *X. laevis* oocytes to characterize their transporter activity (**Fig. 3**). Introduction of a stop codon at Arg240 (R240X) completely abolished the transport activity of SLC6A19, as did the amino acid substitutions E501K and L242P. The locations of these mutations on the predicted protein structure and the sequence context are shown in **Figure 4**. Among the mutated amino acids, Leu242, Arg240 and Glu501 are highly conserved throughout the solute carrier 6 (SLC6) family (data not shown) and their alteration would be expected to affect function. In contrast, Val252 is not conserved in the SLC6 family, consistent with the observed maintenance of transport function in the V252I mutant protein. The common D173N mutation consistently reduced

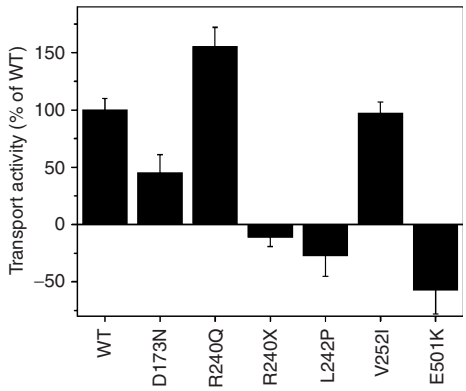

Figure 3 Leucine transport activities in mutant SLC6A19 proteins. Data are presented as a percentage of the activity measured in oocytes expressing wild-type SLC6A19 (WT). Expression of the mutations R240X, L242P and E501K in oocytes partially suppressed the oocyte endogenous leucine uptake activity, resulting in negative transport activities. The mutations R240Q and V252I retained transport activity and did not cosegregate with Hartnup disorder, whereas the mutations D173N, R240X, L242P and E501K are associated with the disease ($n = 5$–9 experiments for each mutant).

transport activity by ~50% but did not completely inactivate the transporter (**Fig. 3**). This residue is not conserved, but a glutamate or aspartate is found at this position in all members of the SLC6 family that actively transport amino acids. A partial reduction of the transport activity in transfected oocytes may reflect impaired transporter activity *in vivo*. Reduced transport activity may result from a trafficking defect that delays expression of the transporter at the cell surface, as observed in the cystinuria-associated mutations (OMIM 220100) of rBAT[22] and in the cystic fibrosis–associated mutations of CFTR (OMIM 602421)[23].

We used real-time quantitative PCR analysis of mRNA obtained from various human tissues to assess the relative abundance of the *SLC6A19* transcript. After normalizing to expression of 18S RNA, we found that the kidney expressed *SLC6A19* more abundantly than the small intestine (0.56 relative abundance), colon (0.045) or prostate (0.085). *SLC6A19* was not detected in testis, whole brain, cerebellum, fetal liver, spleen, skeletal muscle, uterus, heart or lung. These results are consistent both with the distribution of the mouse ortholog[17] and with the fact that >50% of 62 deposited expressed-sequence tags

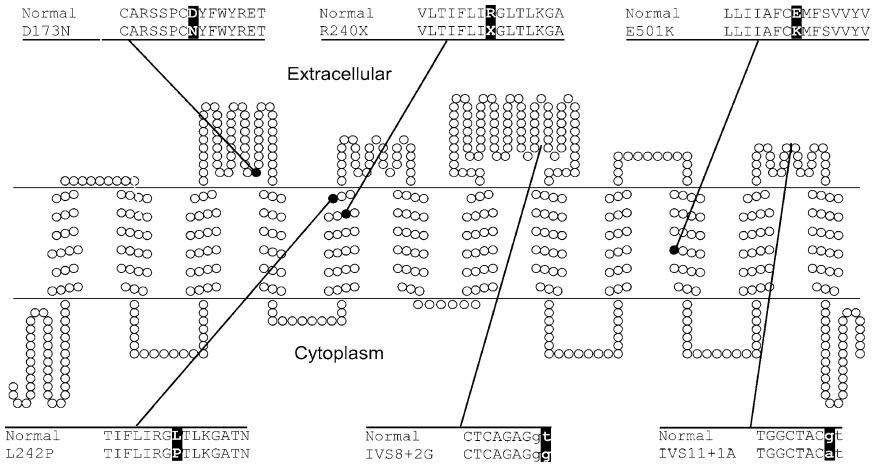

Figure 4 Schematic representation of mutations in the system B^0 transporter SLC6A19. The predicted topology in the membrane suggests that both the N and the C terminus are intracellular and that there are twelve transmembrane domains separating extracellular and cytoplasmic loops as labeled. In mutations associated with Hartnup disease, the three predicted amino acid changes (D173N, L242P and E501K) and single predicted premature stop (R240X) are indicated as black circles, and the two predicted splice variant sites (IVS8+2G and IVS11+1A) are indicated at wild-type exon boundaries. Normal indicates wild-type sequence.

corresponding to the mouse ortholog were identified from kidney and small intestine.

Here we explain the biochemical phenotype of Hartnup disorder through positional, functional and mutational analyses of individuals from seven pedigrees with the disorder that carry mutations in *SLC6A19*, encoding the system B^0 transporter. We confirmed the biochemical phenotypic abnormality imposed by mutations in *SLC6A19* by showing that the mutations identified in pedigrees with Hartnup disorder lead to diminished neutral amino acid transport function *in vitro*. We did not identify an individual with complete *in vitro* inactivation of *SLC6A19* from the ten asymptomatic individuals with urinary neutral hyperaminoaciduria that we studied. This raises the possibility that the diverse clinical signs and symptoms of Hartnup disorder, such as cerebellar ataxia and photosensitive rash, might be limited to individuals who inherit two null alleles; this may explain the reduced clinical penetrance observed. Hartnup disorder presents a similar population profile to that of cystic fibrosis, in which there is a frequent disease-associated allele, which may result in homozygosity. Many affected individuals are compound heterozygotes with respect to the main disease-associated allele (in this case, 517A), carrying a rare or private mutation in the other disease-associated allele. Although the allelic, and consequent functional, heterogeneity of *SLC6A19* may explain the biochemical and clinical phenotypic variation in pedigrees with Hartnup disorder, other loci affecting or modifying the Hartnup phenotype may yet be identified[24,25].

METHODS

DNA preparation and linkage analysis. The pedigrees of six families with Hartnup disorder and approval to study them from the Human Ethics Committee were described previously[9]. We also studied a pedigree containing one affected daughter and her parents. This study conformed to the guidelines of the National Health & Medical Research Council of Australia for the conduct of genetic registers. We extracted DNA according to standard protocols. We chose microsatellites based on proximity to genes or regions of interest in five locations around the genome, as well as heterogeneity. Genotyping was done at the Australian Genome Research Facility. We used a panel of 50 healthy blood donors to establish population allele frequencies for linkage analysis of microsatellites. We carried out pairwise and multipoint linkage analyses using Genehunter 2.1.

Cloning of cDNA. We used human kidney Marathon-Ready cDNA (5 ng; BD Biosciences Clontech) as a template to amplify the *SLC6A19* cDNA using DyNAzyme EXT (0.5 units; Finnzymes). Primer sequences are available on request. The thermocycling conditions were as follows: denaturation at 94 °C for 1 min; followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and polymerization at 72 °C for 90 s; and then a final extension at 72 °C for 10 min. We excised the amplicon from a 1% low-melting agarose gel, ligated it to pGEM-T Easy vector and then transformed it into competent DH5α bacteria. We purified plasmid DNA by anion exchange column (Qiagen) and sequenced it as described in the next section. We predicted the two-dimensional topology model of SLC6A19 using TMHMM 2.0, Tmpred and TOPO2.

PCR amplification and sequencing of exons in *SLC6A19*. We designed oligonucleotide primers used for amplification of the 12 exons of *SLC6A19* to span intron-exon boundaries using Primer 3. Primer sequences are available on request. We purified amplicons using a spin column method with Sephadex G-50 (Sigma). We carried out sequencing reactions using the ABI Prism Big-Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and analyzed the sequences on an ABI Prism 377 DNA Sequencer (SUPAMAC).

Allele frequency determination. We determined the allele frequencies of mutations detected in the coding region of *SLC6A19* in Hartnup disorder probands by analysis of 580 healthy controls of European descent. If the mutated allele resulted in a sequence change that could be detected by a restriction endonuclease, then we designed a PCR-based diagnostic test to detect that mutation. Where possible, we designed the PCR product to include a control site for digestion that could be readily visualized using agarose electrophoresis. If no restriction site was altered by the mutation, we estimated normal allele frequencies by sequencing.

Site-directed mutagenesis. We introduced six single point mutations identified in individuals with Hartnup disorder into *SLC6A19* cDNA using the Quick-change site directed mutagenesis kit (Stratagene). Primer sequences are available on request. All mutations were verified by sequencing (Australian National University, Biomolecular Resource Facility).

Oocytes and injections. Oocyte isolation and management were described previously[26]. For expression, we linearized the oocyte expression vector pGem-He-Juel[27] containing human *SLC6A19* with *Sal*I and transcribed it *in vitro* using the mMessage mMachine Kit (Ambion). We injected oocytes with 20 ng of human *SLC6A19* cRNA or its site-specific mutants and measured transport 3–5 d later. Owing to the low transport activity of human SLC6A19, we used oocytes injected with water instead of uninjected oocytes as controls.

Flux measurements in oocytes. We carried out uptake experiments in ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES buffer; titrated with NaOH to pH 7.4). In sodium-free incubation buffer, we replaced NaCl with NMDG-Cl or LiCl; in chloride-free buffer, we replaced NaCl with sodium gluconate. Uptake of ^{14}C-phenylalanine or ^{14}C-leucine increased linearly with time for up to 20 min. As a result, we determined uptake using an incubation period of 15 min. To determine substrate specificity, we used the following compounds at a concentration of 100 μM: U-^{14}C-phenylalanine, U-^{14}C-glutamine, U-^{14}C-alanine, U-^{14}C-leucine, U-^{14}C-arginine and U-^{14}C-glutamate (Amersham Pharmacia Biotech).

Relative expression level of *SLC6A19* cDNA in human tissue. We detected the expression of *SLC6A19* transcripts in various human tissues by quantitative RT-PCR using a human total RNA Master Panel II (BD Clontech). Total RNA (1 μg) was reverse transcribed at 37 °C with 200 U Moloney murine leukemia virus reverse transcriptase in a volume of 20 μl reaction mixture containing 1 × reaction buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$, 50 mM dithiothreitol), 1 U RNase inhibitor and 0.5 mM dNTPs, using 0.25 μg μl^{-1} oligo$(dT)_{15}$ primer. All reagents were from Promega. We terminated the reaction at 70 °C for 10 min. We amplified 2 μl of cDNA using primers XTF1 and XTR2 (sequences available on request). We used an abundantly expressed gene, 18S, as the internal control to normalize the amount of starting RNA used for RT-PCR for all samples. To determine real-time PCR efficiencies of both *SLC6A19* and 18S, we added twofold serial dilutions of reverse-transcribed total RNA as PCR template. We did PCR in duplicates in 25 μl of reaction buffer containing 12.5 μl of HotStar Taq master mix (Qiagen) and 500 nM of each primer. We used the following protocol: (i) denaturation program (15 min at 95 °C); (ii) amplification and quantification program, repeated 35 cycles for 18S or 45 cycles for *SLC6A19* (20 s at 95 °C, 20 s at 54 °C, 30 s at 72 °C); (iii) melting curve program (60–99 °C with a heating rate of 0.1 °C s^{-1} and fluorescence measurement). We verified the presence of specific product by agarose gel electrophoresis. We calculated the relative expression ratio (R) of *SLC6A19* in tissue RNA (sample) versus kidney RNA (control) using comparative Ct values in the following equation: $R = 2^{-\Delta\Delta Ct(\text{sample} - \text{control})}$ where $-\Delta\Delta Ct\,(\text{sample} - \text{control}) = (Ct\ SLC6A19 - Ct\ \text{of}\ 18S)_{\text{sample}} - (Ct\ SLC6A19 - Ct\ \text{of}\ 18S)_{\text{control}}$. The Ct value is the cycle number at which the gene amplified beyond the threshold. We plotted Ct values versus cDNA concentration input (100, 50, 25, 12.5, 6.25, 3.125, 1.5625 ng) to calculate the slope. We calculated the corresponding real-time PCR efficiencies of *SLC6A19* and 18S according to the equation $10^{(-1/\text{slope})}$.

URLs. TMHMM 2.0 is available at http://www.cbs.dtu.dk/services/TMHMM-2.0/. Tmpred is available at http://www.ch.embnet.org/software/TMPRED_form.html. TOPO2 is available at http://www.sacs.ucsf.edu/TOPO/topo.html. Primer 3 is available at http://frodo.wi.mit.edu/primer3/primer3_code.html.

GenBank accession numbers. *SLC6A19* cDNA, AY591756; existing predicted cDNA, XM_291120.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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