NPT2a gene variation in calcium nephrolithiasis with renal phosphate leak

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A decrease in renal phosphate reabsorption with mild hypophosphatemia (phosphate leak) is found in some hypercalciuric stone-formers. The *NPT2a* gene encodes a sodium-phosphate cotransporter, located in the proximal tubule, responsible for reclaiming most of the filtered phosphate load in a rate-limiting manner. To determine whether genetic variation of the *NPT2a* gene is associated with phosphate leak and hypercalciuria in a cohort of 98 pedigrees with multiple hypercalciuric stone-formers, we sequenced the entire cDNA coding region of 28 probands, whose tubular reabsorption of phosphate normalized for the glomerular filtration rate (TmP/GFR) was 0.7 mmol/l or lower. We performed genotype/phenotype correlations for each genetic variant in the entire cohort and expressed *NPT2a* variant RNAs in *Xenopus laevis* oocytes to test for cotransporter functionality. We identified several variants in the coding region including an in-frame 21 bp deletion truncating the N-terminal cytoplasmic tail of the protein (91del7), as well as other single-nucleotide polymorphisms that were non-synonymous (A133V and H568Y) or synonymous. Levels of TmP/GFR and urine calcium excretion were similar in heterozygote carriers of *NPT2a* variants compared to the wild-type (wt) homozygotes. The transport activity of the H568Y mutants was identical to the wt, whereas the N-terminal-truncated version and the 91del7 and A133V mutants presented minor kinetic changes and a reduction in the expression level. Although genetic variants of *NPT2a* are not rare, they do not seem to be associated with clinically significant renal phosphate or calcium handling anomalies in a large cohort of hypercalciuric stone-forming pedigrees.

Kidney International (2006) **69,** 2261–2267. doi:10.1038/sj.ki.5000437; published online 10 May 2006

KEYWORDS: phosphaturia; hypercalciuria; NPT2a; SLC34A1; nephrolithiasis; polymorphisms

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Received 13 December 2004; revised 13 October 2005; accepted 6 January 2006; published online 10 May 2006

Idiopathic hypercalciuria is the most frequent metabolic disorder associated with calcium nephrolithiasis.[1,2] Family-based,[1,3,4] twin[5] and case–control[6] studies suggest that urine calcium excretion is a heritable trait. Idiopathic hypercalciuria is usually associated with increased intestinal calcium absorption and a subset of patients has a primary mild phosphaturic state, or 'phosphate leak'.[2,7] This results in a compensatory increase in $1,25(OH)_2D_3$ production and higher intestinal phosphate as well as calcium absorption and excretion. The combined phosphaturic and hypercalciuric state favors the crystallization of calcium phosphate salts that later grow into concretions.

Phosphate wasting with nephrolithiasis occurs in several Mendelian traits including Dent disease[8] and hereditary hypophosphatemic rickets with hypercalciuria.[9] *NPT2a* encodes the rate-limiting proximal tubule sodium phosphate cotransporter (reviewed by Murer *et al.*[10]) and is thus a candidate gene for hereditary renal phosphaturia. A previous study has associated *NPT2a* variants with phosphate leak in two of 20 subjects, with either osteoporosis or recurrent nephrolithiasis,[11] possibly through dominant-negative effects. However, this study had several limitations and was recently criticized.[12] First, although *NPT2a* variants were suggested to be functional by demonstration of decreased phosphate currents and sodium-dependent phosphate uptake *in vitro* (including when coexpressed with the wild type (wt)), the currents were rather small. Second, as the pedigrees were not sampled, there was no segregation analysis of *NPT2a* variants or a population association study to confirm that other carriers had abnormal renal phosphate handling. Finally, there was no attempt made to determine the population prevalence of mutant *NPT2a* in a cohort of stone formers or osteoporotic subjects.

To determine whether genetic variants of *NPT2a* cause renal phosphate handling anomalies in a large cohort of families with multiple and recurrent stone-forming subjects, we sequenced the entire coding region of *NPT2a* in siblings with a low tubular reabsorption of phosphate normalized for the glomerular filtration rate (TmP/GFR). We further evaluated the association between genetic variants and TmP/GFR by genotyping extended pedigrees. We also conducted electrophysiological studies of *NPT2a* variants in *Xenopus laevis* oocytes. Our findings demonstrate that

NPT2a variants are neither rare nor responsible for renal phosphate leak in the population tested.

RESULTS

Sequencing and genotyping

We identified a total of five polymorphisms, of which three were non-synonymous variants (Table 1 and Figures 1 and 2) in three different probands. These included a 21 bp in-frame deletion (91del7) in the 5′-end corresponding to amino acids 91–97 (VPKLRQA) in the N-terminal intracellular segment. The deletion polymorphism was identified in all three subjects by sequencing after polymerase chain reaction (PCR) amplification of the appropriate region and subcloning into the pCR2.1 vector (Stratagene, La Jolla, CA, USA). Restriction enzyme digestion with *Bgl*I also allowed the identification of carriers of the deletion polymorphism (Figure 1d). The H568Y variant in exon 13 was also confirmed by subcloning and detection with *Kpn*I digestion (Figure 2b). It was present in the same clones, that is, on the same haplotype as the 91del7 deletion. The A133V variant was found in a single proband.

Table 1 | Genetic variants of *NPT2a* identified in 28 stone-formers with low TmP/GFR (less than 0.7 mmol/l)

Exon	Polymorphism	Number of chromosomes
4	91del7 21 bp in-frame deletion GTCCCCAAGCTGCGCCAGGCT 352–372 of cDNA	3
5	C479T (A133V)	1
7	T855C (conserved)	13
13	C1783T (H568Y)	5
3′ UTR	G2180A	1

bp, base pair; TmP/GFR, tubular reabsorption of phosphate normalized for the glomerular filtration rate; UTR, untranslated region.

Phenotypes in extended pedigrees

We used *Bgl*I digestion to identify carriers of the deletion in extended pedigrees and compared phenotypes. As shown in Table 2, TmP/GFR, serum phosphate levels, urine calcium

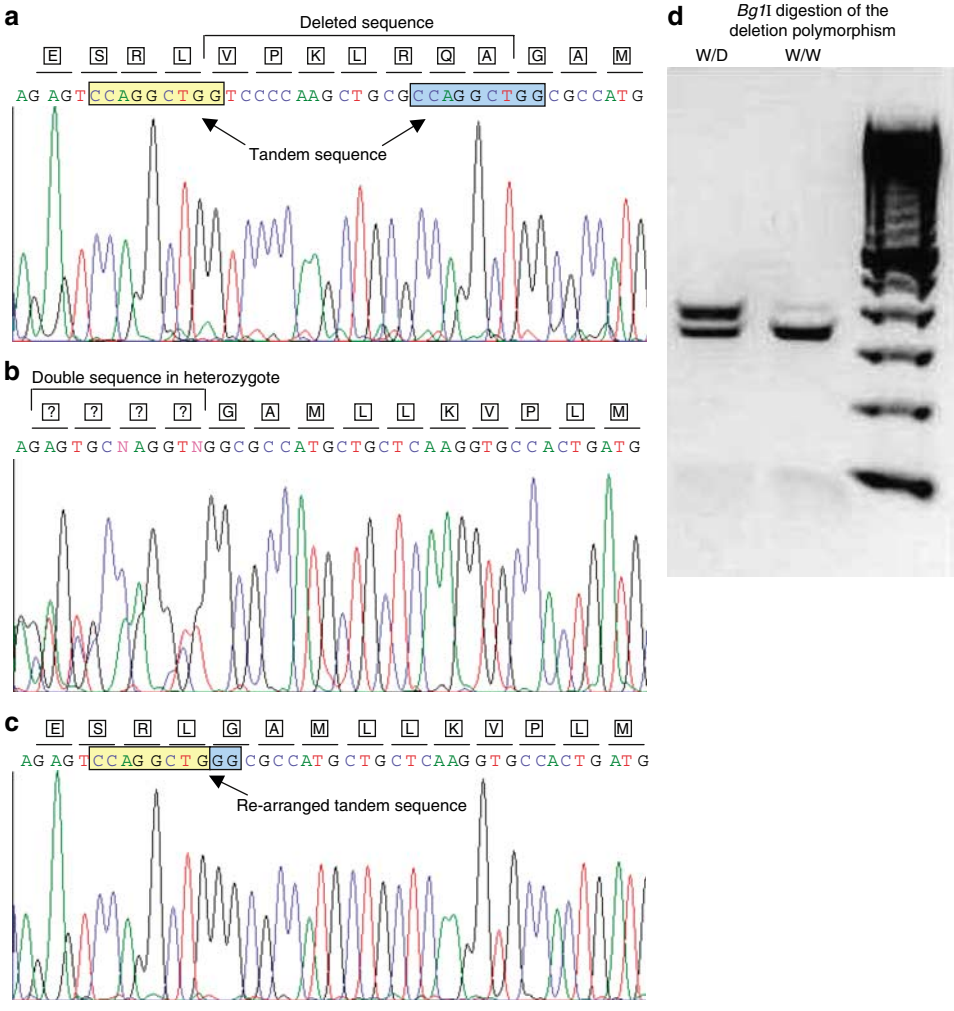

Figure 1 | *NPT2a* gene sequencing of exon 4. (**a**) Wt sequence of exon 4 showing position of the 21 bp deletion and flanking tandem repeat sequences responsible for strand slippage. (**b**) Sequence of a heterozygote carrier of the deletion mutant showing double sequence at the start of the deletion. (**c**) Sequencing of the cloned *NPT2a* deletion variant. (**d**) *Bgl*I digestion of the deletion polymorphism in a W/91del7 heterozygote and wt homozygote.

excretion, and number of stone episodes were similar in heterozygotes compared to wt homozygotes. Serum parathyroid hormone levels were marginally higher in heterozygotes.

Population screening of the *NPT2a* variants

To determine whether *NPT2a* variants are present in an unselected population, we screened 96 anonymous DNA samples by PCR from a Caucasian North American population and identified six heterozygotes for the deletion, three of which were also heterozygous for H568Y (data not shown). We identified one subject with the H568Y variant only.

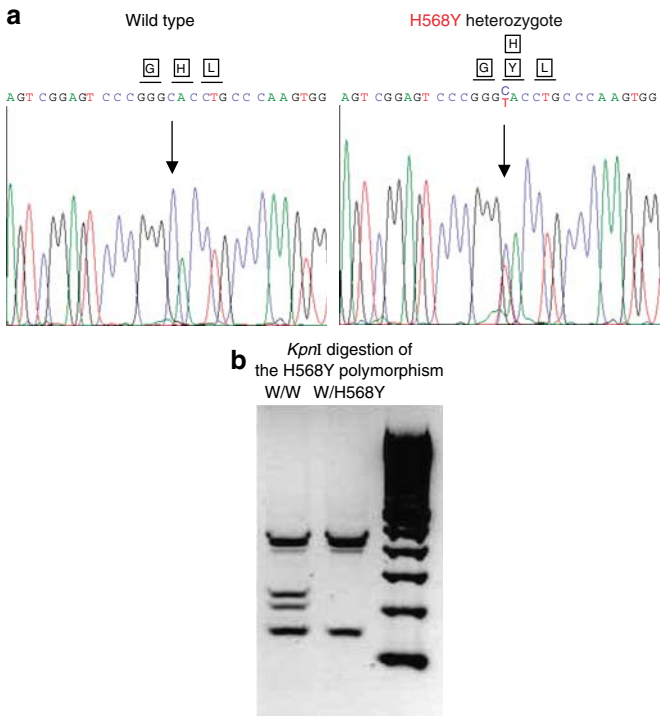

Figure 2 | *NPT2a* gene sequencing of exon 13. (**a**) *NPT2a* gene sequencing of exon 13 showing the wt sequence and a heterozygote H568Y carrier. (**b**) *Kpn*I digestion of a wt homozygote and W/ H568Y heterozygote.

Expression of NPT2a in *X. laevis* oocytes

Wt NPT2a was expressed in *Xenopus* oocytes as were four different variants (the A133V, the N-terminal deletion 91del7, H568Y, and 91del7-H568Y, which contains both variants). Examples of currents recorded in the presence of Barth's solution and after addition of 1 mM Pi are shown in Figure 3a. The inorganic phosphate (Pi)-dependent current–voltage relationship (Figure 3b) is roughly linear and non-injected oocytes were completely insensitive to Pi addition, in agreement with previous observations.[13] At −50 mV, Pi-induced currents through the different NPT2a proteins are shown in Figure 3c. There was no difference between the levels of transport mediated through wt NPT2a and the H568Y mutant, but lower levels of current were observed for the A133V mutants ($P<0.05$) and for the 91del7 and 91del7-H568Y mutants ($P<0.001$).

A Pi concentration of 1 mM corresponds to a saturating concentration for the wt cotransporter as its reported affinity constant at pH 7.4 is 0.11 mM.[12] Pi affinity constants, roughly estimated by comparing the currents stimulated by the addition of 0.1 and 1 mM Pi, were always between 0.05 and 0.10 mM for A133V, 91del7, H568Y, and 91del7-H568Y, and were not significantly different from the wt affinity constants (data not shown). Consequently, the observed reduction in the Pi-dependent currents for A133V, 91del7, and 91del7-H568Y could not be owing to a large increase in the Pi affinity constant and it must either be owing to a reduced turnover rate of A133V, 91del7, and 91del7-H568Y or to a reduction in the number of expressed cotransporters. Discrimination between these two possibilities was possible by examining transient currents expressed by these proteins.

Transient (or presteady-state) currents are thought to reflect the presence of mobile charges, associated with each cotransporter, which move within the membrane electrical field.[14,15] These currents can be modelled by a Boltzmann equation from which three characteristic parameters were extracted: Q_{max}, $V_{0.5}$, and z. Q_{max} is the total mobile charge, $V_{0.5}$ is the membrane potential at which the mobile charge is equilibrated between the inward and outward facing configuration, and z is the equivalent valence of a charge that moves across the entire membrane electric field.

Table 2 | Genotype–phenotype correlations for the 91del7 *NPT2a* gene variant in 722 siblings from 98 families

	W/W		W/91del7[a]		
	Males (303)	Females (348)	Males (36)	Females (35)	*P*-value
Age	52.2±14.2	53.2±14.4	50.7±10.9	47.9±15.0	0.54
Stone-formers (%)	192 (63.4)	165 (47.4)	24 (66.7)	15 (42.9)	0.99
SPO_4 (mmol/l)	1.06±0.24	1.13±0.25	1.02±0.18	1.09±0.16	0.25[b]
TmP/GFR (mmol/l)	0.81±0.25	0.90±0.29	0.77±0.21	0.88±0.14	0.35[b]
UCaV (mmol/day)	6.8±3.0	5.2±2.3	8.3±4.8	5.3±2.0	0.11
UCaV/kg (mmol/kg/day)	0.09±0.04	0.08±0.04	0.10±0.05	0.08±0.04	0.11
SCa (mmol/l)	2.34±0.10	2.33±0.09	2.36±0.11	2.35±0.10	0.05
PTH (pmol/l)	3.78±2.39	3.68±2.25	3.31±1.53	2.95±1.21	0.03

PTH, parathyroid hormone; SCa, serum calcium; SPO, serum phosphate; TmP/GFR, tubular reabsorption of phosphate normalized for the glomerular filtration rate.
All values mean±s.d.
[a]Includes subjects with haplotype 91del7/H568Y.
[b]*P*-values corrected for gender.

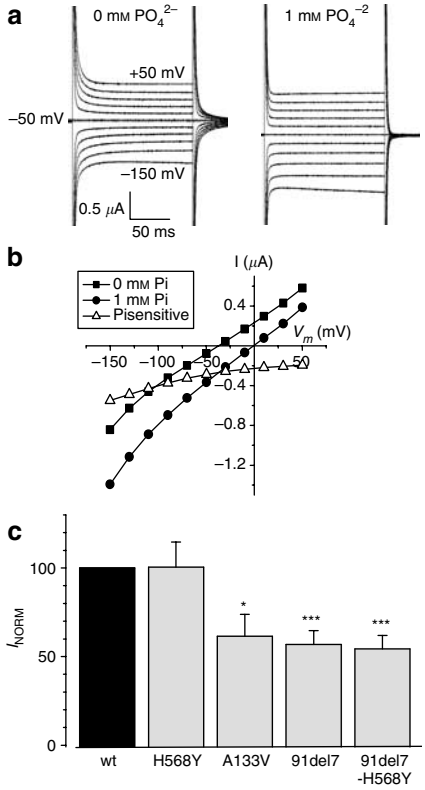

Figure 3 | Phosphate-induced current in *X. laevis* oocytes injected with wt or variant NPT2a cRNA. (**a**) Examples of currents recorded in the absence or in the presence of 1 mM HPO_4^{2-}. The *I–V* curves obtained from these currents are presented in (**b**) together with Pi-induced current obtained by subtracting one current from the other. (**c**) The normalized Pi-induced current at −50 mV (±s.e.m.) for the four variants (H568Y, A133V, 91del7, and 91del7-H568Y) with respect to the wt Na/Pi cotransporter ($n = 16$–18 oocytes from three to five different donors for each column). The currents were found significantly lower than for wt cotransporters in the case of A133V (* or $P < 0.05$) and in the case of 91del7 and 91del7-H568Y (*** or $P < 0.05$).

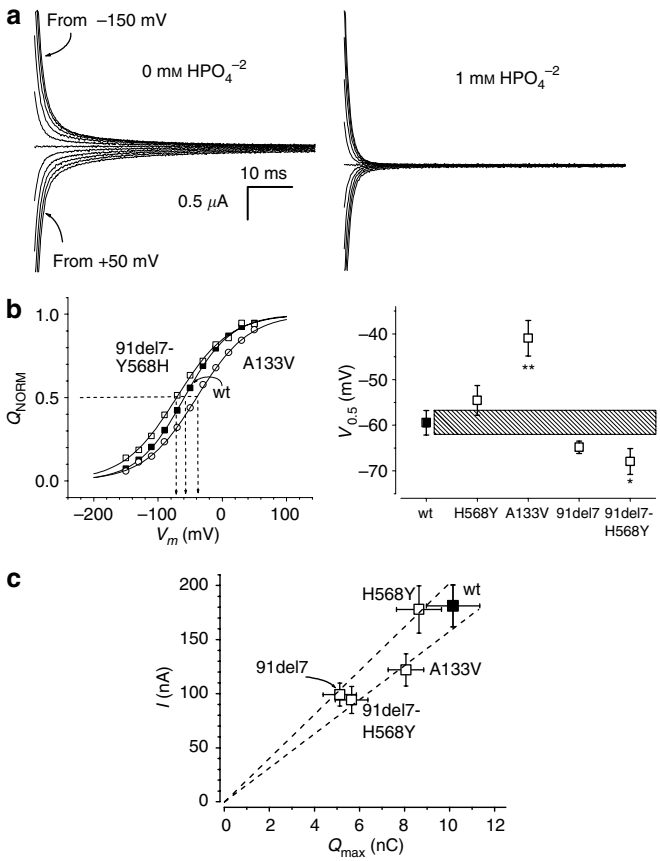

Figure 4 | Transient currents recorded from the NPT2a cotransporter. (**a**) An example of transient currents from an oocyte expressing the wt NPT2a cotransporter. These tail currents were recorded 2 ms after return of the membrane potential to −50 mV from various potentials ranging from −150 to +50 mV. It is evident that the presence of 1 mM Pi greatly inhibits these transient currents. The charge transferred is obtained from the area under the current traces and the portion specifically related to the cotransporter can be obtained by subtraction of the residual charge observed in the presence of Pi. In (**b**), this is performed for typical oocytes expressing the wt cotransporter, the A133V mutant and the 91del7-H568Y mutant. The *Q* vs *V* curves are fitted with the Boltzman equation and normalized to the maximal charge. The $V_{0.5}$ values for the H568Y mutant (−55 ± 3 mV, $n = 13$) and the 91del7 mutant (−65 ± 2 mV, $n = 11$) were not found to be statistically different from the $V_{0.5}$ value of the wt cotransporter (−59 ± 3 mV, $n = 25$). In contrast, the $V_{0.5}$ values of the 91del7-H568Y mutant (−68 ± 3 mV, $n = 14$) for the A133V mutant (−41 ± 4 mV, $n = 14$) (were found to be significantly different from the wt $V_{0.5}$ ($P < 0.05$, and $P < 0.01$, respectively). In (**c**), the correlation between the steady-state Pi-induced current (1 mM HPO_4^{2-}) and the maximal charge transferred is shown for the (■) wt NPT2a and the (□) four variants.

In Figure 4a, examples of transient currents are shown where the membrane voltage is returned to −50 mV from potentials ranging from −150 to +50 mV. Under our experimental conditions, 99% of a 100 mV pulse is achieved within 2 ms and most of the large capacitive current can be eliminated by considering the transient current remaining after this initial period. These transient currents are not present in non-injected oocytes and vanish in the presence of a saturating Pi concentration (Figure 4a). In Figure 4b, normalized *Q* is displayed as a function of the conditioning membrane potential for the wt cotransporter and for the 91del7-H568Y and the A133V mutants. The valence of the mobile charge associated with NPT2a did not change significantly between the wt (0.67), A133V (0.76), 91del7 (0.67), H568Y (0.72), and 91del7-H568Y (0.70). This is consistent with previous findings for wt NPT2a and for a few NPT2a mutants.[16] In contrast, a small but significant negative shift in $V_{0.5}$ was observed for the 91del7-H568Y mutant ($P < 0.05$) (see Figure 4b), whereas the $V_{0.5}$ values for the A133V was observed to be about 20 mV more positive than for the wt ($P < 0.01$) (see Figure 4b).

Q_{max} correlated closely with the steady-state current owing to the addition of 1 mM Pi (Figure 4c). Q_{max} averaged 10.1 ± 1.2 and 8.6 ± 1.0 nC for the wt and H568Y, whereas it was reduced to 8.1 ± 0.8 nC for A133V, to 5.1 ± 0.7 nC for 91del7, and to 5.6 ± 0.7 nC for 91del7-H568Y. In the absence of a significant change in the '*z*'-value, this is consistent with the simple interpretation that V133C, 91del7, and

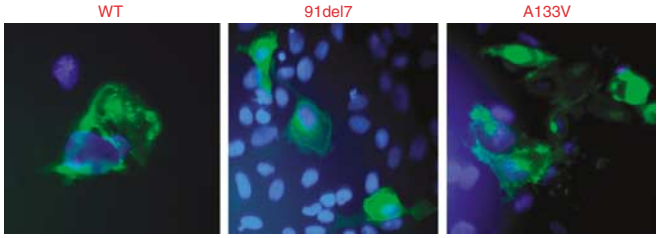

Figure 5 | Expression of wt and mutant cotransporters in OK cells. Cells were transfected with the EGFP-fused wt and mutant cotransporters and processed for deconvolution fluorescence microscopy.

91del7-H568Y display a lower Pi-induced current because their expression levels are lower compared with the wt and the H568Y mutant.

Expression of NPT2a in kidney cells

As shown in Figure 5, enhanced green fluorescent protein (EGFP)-tagged NPT2a variants were properly expressed when transfected in OK cells, a cell line with proximal tubular characteristics. The accumulation of cotransporters could be seen as dots in the cytoplasm and near the plasma membrane surface. Expression levels estimated by Western blotting were similar (data not shown).

DISCUSSION

This study demonstrates that, in a large cohort of hypercalciuric pedigrees ascertained for the presence of multiple kidney stone formers, genetic polymorphisms at the *NPT2a* locus are not associated with variation in renal phosphate handling. We carefully selected index cases with decreased renal phosphate reabsorption and sequenced the entire coding region of the *NPT2a* gene. We identified several variants, one of which leads to a 7-amino-acid deletion in the N-terminal region of the transporter (91del7). When extended pedigrees were examined, there were no differences in serum phosphate, TmP/GFR, or urine calcium excretion between carriers and non-carriers of genetic variants. In addition, these variants were identified in a random population. However, we did not find 91del7 homozygotes, which could potentially present with a clinical phenotype of mild phosphate wasting. Heterologous expression in *X. laevis* oocytes demonstrated similar phosphate transport characteristics for the NPT2a variants. It remains to be determined whether the lower expression levels observed for variants containing 91del7 and A133V have any implication for their *in vivo* expression. Incidentally, we subsequently found that the deletion variant and exon 13 substitution have been described by a human complementary DNA sequencing consortium[17] as 'highly similar to NPT2' (GI Accession number: 21755493). Therefore, our results confirm that those are *NPT2a* genetic variants and not splicing variants or distinct NPT2a isoforms.

The 91del7 deletion located in the N-terminus probably arose from DNA strand slippage. This would have occurred by pairing between direct repeats of homologous sequences of 5 bp[18,19] that flank the deletion (Figure 1). The role of the intracellular N-terminal region of NPT2a is unknown and measurable consequences of the 91del7 and A133V variants were examined clinically and for functional transport or expression consequences *in vitro*. Although these genetic variants of *NPT2a* are not rare, they do not lead to significant phenotypic differences either *in vivo* or *in vitro*. In addition, it is unlikely that other functional NPT2a variants are frequently associated with renal phosphate handling anomalies in hypercalciuric stone-formers, at least in our population. We speculate that other genetic variants such as identified by Prié *et al.*[11] are rare. Furthermore, the functionality of such variants has been questioned as *in vitro* findings in this study were not subsequently confirmed.[12]

It has been suggested that serum phosphate and, possibly, renal phosphate handling are genetically determined.[5] The *NPT2a* gene is a legitimate candidate as it encodes the apical membrane sodium phosphate cotransporter located in proximal tubules. In mice, targeted disruption of the *NPT2* gene ($Npt2^{-/-}$) leads to hypophosphatemic rickets,[20] and although heterozygous $Npt2^{+/-}$ mice have a normal serum phosphate concentration, renal phosphate excretion is significantly increased compared with wt mice. These characteristics resemble those found in the subjects selected in our study. Finally, no mutations in *NPT2a* were found in a large Bedouin pedigree or in four other small families with hereditary hypophosphatemic rickets with hypercalciuria.[21] Other disorders associated with phosphaturia include hypophosphatemic rickets owing to mutations in PHEX and FGF23. These diseases are not associated with nephrolithiasis and phosphaturia is thought to result from decreased protein expression and insertion of NPT2 in the brush-border membrane. NHERF-1, encoding a protein adapter that participates in the hormone-induced internalization of NPT2, is another potential candidate as mice in which this gene was deleted exhibit renal phosphate wasting with hypercalciuria.[22] However, renal phosphate excretion does not seem to correlate with urine calcium excretion.[23]

In contrast to simple Mendelian disorders, the identification of genes causing complex traits such as idiopathic hypercalciuria represents a formidable challenge. Performing candidate gene studies in subgroups of patients with particular phenotypes such as mild phosphate wasting is a more targeted approach with a higher potential yield for gene discovery. We have previously excluded several candidate genes using this approach.[24–26] However, a more global approach will eventually be required. For example, the identification of common variants of proteins whose function is known, by projects such as the HapMap initiative,[27] will facilitate gene discovery for complex traits.

In conclusion, *in vivo* and *in vitro* studies do not support a role for *NPT2a* gene variation in 'phosphate leak' associated with calcium nephrolithiasis.

MATERIALS AND METHODS

Families and probands

This study was approved by institutional review committees, and informed consent was obtained from all participants. A complete description of the study design has been published.[1] From September 1995 to February 2004, we recruited families of French-Canadian descent from specialized stone clinics and lithotripsy units. These clinics ascertain approximately 1000 patients annually. Questionnaires were systematically handed to probands. Subjects with at least one episode of urolithiasis of predominant calcium content, as determined by crystallographic analysis (Louis C Herring & Co., Orlando, FL, USA), and a positive family history in a sibling, were interviewed by a clinical research nurse and asked to contact affected and unaffected siblings. Families with at least two affected sibs were followed up. Thereafter, all consenting affected siblings from the same sibship were interviewed and phenotyped on the same day and place, on an outpatient basis. Urine sampling consisted of 24-h urine collection under free dietary conditions as well as a fasting morning void. Serum was also obtained for biochemical analyses. We attempted to recruit all affected subjects, but elected to ascertain up to two unaffected sibs per sibship. Affected siblings had at least one stone episode verified by crystallographic analysis or by an imaging method consisting in intravenous pyelogram or abdominal ultrasound. Siblings with no stone passage but with an imaging method showing a stone were also considered affected. Episodes and onset age of urolithiasis for sibs and probands were confirmed by a detailed review of all records available from the respective hospitals where clinical assessments were made. Siblings with medical conditions predisposing to urolithiasis (hyperparathyroidism, inflammatory bowel disease, chronic diarrhea, urinary tract infection before stone episodes) or on medications susceptible to modify urine composition (diuretics, calcium, and vitamin supplements) were not included in the study. A total of 98 hypercalciuric pedigrees were included in the study comprising 722 relatives. From those, we selected 28 stone-forming sibs with a threshold for phosphate reabsorption (TmP/GFR) of 0.7 mmol/l or lower. Clinical characteristics of the cohort and the 28 index cases are shown in Tables 3 and 4, respectively.

Sequencing of the *NPT2a* gene

We used intronic primers to amplify all 13 exons of the *NPT2a* gene from the 28 index cases (sequences available upon request). Amplification was performed for 35 cycles using standard PCR methods. The PCR products were sequenced using a commercially available kit (BigDye Terminator 3.0 Cycle Sequencing Kit from Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 3100 automated sequencer (Applied Biosystems). Both DNA strands were sequenced and analyzed using the appropriate software (Applied Biosystems). Sequence alignments were performed with bl2seq (http://www.ncbi.nlm.nih.gov/BLAST) and CLUSTALW (http://bioweb.pasteur.fr) online software.

When possible, restriction enzyme digestion of amplified genomic DNA was used for subsequent confirmation or identification of individual genotypes.

Expression of NPT2a in *X. laevis* oocytes

In vitro transport characteristics of the genetic variants identified in three families were analyzed by expressing the mutated RNA in *X. laevis* oocytes. Wt complementary DNA encoding human NPT2a (kindly provided by Drs J Biber and H Mürer, Physiology Institute, University of Zurich, Zurich, Switzerland) was introduced into the transcription vector pSP64T between the *Xho*I and *Not*I sites. Specific genetic variants were introduced into the wt cDNA using a commercially available kit (Stratagene), and confirmed by dideoxy sequencing. Capped mRNA was synthesized *in vitro* using the mMessage mMachine kit (Ambion, Austin, TX, USA) with SP6 RNA polymerase. Purified RNA was quantified by absorption at 260 nm, and the quality was verified by calculating the ratio of readings at 260 and 280 nm (A_{260}/A_{280}). Preparation of defolliculated *X. laevis* oocytes (stages V or VI) was performed as described previously.[28,29] For wt or variant NPT2a mRNA expression, oocytes were injected with 46 nl of solution containing 0.1 $\mu g/\mu l$ of mRNA in water. They

Table 4 | Clinical characteristics for 28 stone-formers selected for low TmP/GFR (less than 0.7 mmol/l)

	Males (19)	Females (9)
Age (years)	54.5 ± 11.5	47.3 ± 11.3
Stone episodes (no.)	18 ± 45	5.3 ± 3.6
Serum PO_4 (mmol/l)	0.72 ± 0.07	0.76 ± 0.07
TmP/GFR (mmol/l)	0.48 ± 0.11	0.51 ± 0.12
24-h UCaV (mmol/day)	5.84 ± 3.12	4.55 ± 2.01
24-h UCaV/kg (mmol/kg/day)	0.071 ± 0.041	0.069 ± 0.030
Serum Ca (mmol/l)	2.28 ± 0.10	2.28 ± 0.09
PTH (pmol/l)	4.23 ± 2.37	4.81 ± 2.32

PTH, parathyroid hormone; SCa, serum calcium; SPO, serum phosphate; TmP/GFR, tubular reabsorption of phosphate normalized for the glomerular filtration rate; UCaV, 24-h urine calcium excretion.
All values mean ± s.d.

Table 3 | Clinical data for 722 siblings from 98 stone-forming and hypercalciuric pedigrees

	Males			Females			
	Non-stone forming (109)	Stone forming (215)	*P*-value	Non-stone forming (178)	Stone forming (180)	*P*-value	Total *P*-value
Age	53.4 ± 15.0	51.2 ± 13.1	0.16	54.5 ± 14.2	50.7 ± 13.8	0.01	0.004
Stone episodes (no.)	—	7.7 ± 20	—	—	3.9 ± 5.7	—	—
SPO_4 (mmol/l)	1.09 ± 0.25	1.04 ± 0.23	0.06	1.14 ± 0.18	1.10 ± 0.29	0.09	0.01[a]
TmP/GFR (mmol/l)	0.84 ± 0.28	0.79 ± 0.24	0.10	0.91 ± 0.26	0.87 ± 0.30	0.20	0.01[a]
UCaV (mmol/day)	5.75 ± 2.4	7.5 ± 3.5	$<10^{-3}$	4.7 ± 2.1	5.7 ± 2.4	0.004	$<10^{-5}$
UCaV/kg (mmol/kg/day)	0.07 ± 0.03	0.10 ± 0.04	$<10^{-3}$	0.08 ± 0.04	0.09 ± 0.04	0.03	$<10^{-5}$
SCa (mmol/l)	2.34 ± 0.10	2.33 ± 0.11	0.85	2.34 ± 0.09	2.33 ± 0.09	0.32	0.54
PTH (pmol/l)	3.43 ± 1.16	3.87 ± 2.56	0.075	3.76 ± 2.49	3.37 ± 1.69	0.10	0.93

PTH, parathyroid hormone; SCa, serum calcium; SPO, serum phosphate; TmP/GFR, tubular reabsorption of phosphate normalized for the glomerular filtration rate; UCaV, 24-h urine calcium excretion.
All values mean ± s.d.
[a]Corrected for gender.

were then incubated for 4–7 days at 18°C in Barth's solution (in mM: 90 NaCl, 3 KCl, 0.82 $MgSO_4$, 0.41 $CaCl_2$, 0.33 $Ca(NO_3)_2$, 5 HEPES, pH 7.6) supplemented with 5% horse serum, 2.5 mM Na^+-pyruvate, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.1 mg/ml kanamycin.

Na/Pi cotransporter activity was assessed using the two-electrode voltage clamp technique[29,30] in the presence of a bathing medium (control solution) containing (in mM): 90 NaCl, 3 KCl, 0.82 $MgCl_2$, 0.74 $CaCl_2$, 10 mM HEPES, pH adjusted to 7.4 with tris[hydroxymethyl]aminomethane. When needed, 1 or 0.1 mM HPO_4^{2-} was added to the bathing solution using a stock solution containing 200 mM KH_2PO_4 and 800 mM K_2HPO_4 at pH 7.44 to obtain the Pi-sensitive steady-state current. Presteady-state currents (the transient currents that persisted after all capacitive currents have vanished, ~2 ms after the onset of a voltage pulse) were also recorded. These currents reflect the charge movement associated with the voltage-dependent conformational changes of the cotransporter,[14,15] which may represent the displacement of the Na binding site across the membrane electrical field.

Expression of NPT2a variants in opossum kidney cells

The wt and mutant *NPT2*a full-length cDNAs previously cloned in the pSPORT vector were amplified by PCR with primers containing the *Xho*I or *Sac*II restriction sites for insertion in the pEGFP-C1 plasmid in-frame with EGFP. OK cells were cultured on tissue culture coverslips in 35 mm Petri dishes in minimum essential medium (Invitrogen, Burlington, ON, Canada) containing 10% fetal bovine serum. At about 80% confluence, cells were transfected with 2 μg of the EGFP–NPT2a constructs using 10 μl of Lipofectamine 2000 (Invitrogen) in a total volume of 500 μl/well of Opti-minimum essential medium I medium (Invitrogen). After 4 h, the transfection medium was replaced with minimum essential medium.

The coverslips were removed 48 h after transfection from the culture dishes and placed in 4% paraformaldehyde for 30 min to fix the cells. The coverslips were then washed five times in phosphate-buffered saline, and placed face down on a microscope slide on a drop of Vectashield mounting medium containing DAPI (4′,6-diamidine-2′-phenylindole dihydrochloride) (Vector Laboratories, Burlington, ON, Canada) to stain the nuclei. The coverslips were sealed with nail polish, and the cells were observed for fluorescence within 4 h. The cells were photographed on a Leica DMR fluorescence microscope using filters for DAPI and EGFP, and the two images were combined using the OpenLab3.1.1 program (Improvision).

Statistical analysis

All continuous variables were analyzed with SPSS statistical software package (Chicago, IL, USA). Bonferroni's correction was applied for multiple testing where appropriate.

ACKNOWLEDGMENTS

This study was supported by a grant from the Canadian Institutes of Health Research (CIHR) to AB. AB is a Scholar of le Fonds de la Recherche en Santé du Québec (FRSQ).

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