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Gain-of-function haplotype in the epithelial calcium channel TRPV6 is a risk factor for renal calcium stone formation

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The rate-limiting step of dietary calcium absorption in the intestine requires the brush border calcium entry channel TRPV6. The TRPV6 gene was completely sequenced in 170 renal calcium stone patients. The frequency of an ancestral TRPV6 haplotype consisting of three non-synonymous polymorphisms (C157R, M378V, M681T) was significantly higher ($P = 0.039$) in calcium stone formers (8.4%; derived = 502, ancestral = 46) compared to non-stone-forming individuals (5.4%; derived = 645, ancestral = 37). Mineral metabolism was investigated on four different calcium regimens: (i) free-choice diet, (ii) low calcium diet, (iii) fasting and (iv) after a 1 g oral calcium load. When patients homozygous for the derived haplotype were compared with heterozygous patients, no differences were found with respect to the plasma concentrations of 1,25-vitamin D, PTH and calcium, and the urinary excretion of calcium. In one stone-forming patient, the ancestral haplotype was found to be homozygous. This patient had absorptive hypercalciuria. We therefore expressed the ancestral protein (157*R*+378V+681T) in *Xenopus* oocytes and found a significantly enhanced calcium permeability when tested by a $^{45}Ca^{2+}$ uptake assay (7.11 ± 1.93 versus 3.61 ± 1.01 pmol/min/oocyte for ancestral versus derived haplotype, $P < 0.01$). These results suggest that the ancestral gain-of-function haplotype in TRPV6 plays a role in calcium stone formation in certain forms of absorptive hypercalciuria.

INTRODUCTION

Kidney stone disease is a major health problem worldwide, with a lifetime incidence of ~10%. Genetic factors appear to be involved since ~40% of these patients have a positive family history. Idiopathic hypercalciuria is the most common abnormality observed in calcium (Ca^{2+}) stone formers (1–3). It has previously been reported that polymorphisms in the vitamin D receptor (VDR) gene are associated with a specific form of absorptive hypercalciuria, causing hyper-activation of intestinal Ca^{2+} absorption (4). Moreover, spontaneous stone-forming rats [genetic hypercalciuria stone-forming (GHS) rats] have higher levels of VDR protein in the small intestine compared to wild-type rats (5). Other reports suggested that Ca^{2+}-sensing receptor (CaR) polymorphisms are involved in hypercalciuria without kidney stones (6). These findings indicate that several genes contribute to hypercalciuria. Interestingly, there is no report thus far indicating an association between hypercalciuria with Ca^{2+} stone formation and genetic variations of the genes responsible for epithelial Ca^{2+} transport, which could directly affect urine Ca^{2+} levels.

The intestinal and renal epithelial Ca^{2+} transport mechanisms are comprised of three steps: (i) apical Ca^{2+} entry via TRPV-calcium channels (transient receptor potential channels, subtype V), which is likely to be a rate-limiting step of transepithelial calcium transport; (ii) binding of Ca^{2+} to calbindin D which serves as an intracellular Ca^{2+} buffer, and; (iii) basolateral calcium exit via the plasma membrane Ca^{2+} pump and/or the Na^+/Ca^{2+} exchanger (7,8). Steps 1 and 2 are induced by

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1,25-dihydroxyvitamin D, the active form of vitamin D. The key molecules for the apical Ca^{2+} entry, TRPV5 and TRPV6, were previously identified by expression cloning from the kidney and small intestine, respectively (9,10). They are epithelial Ca^{2+}-selective channels with six transmembrane domains and a pore region. *Trpv*5 knockout mice exhibited renal leak hypercalciuria with increased intestinal Ca^{2+} absorption due to a compensatory upregulation of *Trpv*6 (11). In humans however, no phenotype–genotype relationship between TRPV5 polymorphisms and hypercalciuria has been observed so far (12), possibly due to this compensation. On the other hand, *Trpv*6 knockout mice exhibited decreased intestinal Ca^{2+} absorption without obvious compensation by *Trpv*5 in the intestine, resulting in secondary hyperparathyroidism (13).

In this study, we investigated the coding region of the TRPV6 gene in 170 Swiss Ca^{2+} stone formers. We found a haplotype containing three non-synonymous polymorphisms. This haplotype has already been described in a recent genetic study where a positive selection during human evolution was suggested (14). Our functional analysis of this haplotype indicates that it produces a gain-of-function channel, suggesting that the ancestral haplotype causes hyper-activation of intestinal Ca^{2+} absorption, which in turn leads to absorptive hypercalciuria.

RESULTS

Identification of polymorphisms in Ca^{2+} stone-forming patients

We investigated polymorphisms in 170 Ca^{2+} stone-forming patients by direct PCR sequencing of the coding region of the TRPV6 gene (primer information is available in Supplementary Material, Table S1). Three major non-synonymous polymorphisms were identified (C157R+M378V+M681T; Table 1 and Fig. 1A), as well as several low incidence, non-synonymous polymorphisms (R138C+T269M, L259Q and A566P; Fig. 1B) and various synonymous polymorphisms (I283I, L292L, P312P, T360T, N464N, T601T, G626G and N694N; Table 1 and Supplementary Material, Table S2). Interestingly, 25 patients had all of the above listed three non-synonymous polymorphisms, suggesting that these polymorphisms represent a set of alleles of a specific haplotype. We therefore focused on this ancestral haplotype, which includes the three non-synonymous polymorphisms (C157R+M378V+M681T), for further analysis.

Table 1. SNPs in the coding region of TRPV6

	Nucleotide	Amino acid	AA exchange	*n*	Comment
Exon 4	c415t	R138C	Arg *f* Cys	1	New
	t471c	C157R	Cys *f* Arg	26	rs4987657[a]
Exon 7	t776a	L259Q	Leu *f* Gln	1	New
	c806t	T269M	Thr *f* Met	1	New
	c849t	I283I		2	New
	g876a	L292L		1	New
Exon 8	g936a	P312P		1	New
	g1080a	T360T		26	rs4987665[a]
Exon 9	a1132g	M378V	Met *f* Val	26	rs4987667[a]
Exon 11	c1392t	N464N		21	rs4987704
Exon 13	g1696c	A566P	Ala *f* Pro	1	New
Exon 14	g1803a	T601T		5	rs4987678
	a1878g	G626G		26	rs4987679[a]
Exon 15	t2042c	M681T	Met *f* Thr	27	rs4987682[a]
	t2082c	N694N		11	rs4987683

n, number of SNPs found in patient cohort.
[a]SNPs belonging to the ancestral haplotype.

Clinical data

Table 2 shows the basal characteristics and clinical data of derived (homozygous for the new haplotype), heterozygous (new/ancestral haplotype) and homozygous (ancestral haplotype) patients. Although there appears to be no difference between phenotypes based on the parameters shown in Table 2, the homozygous patient had more stone episodes (>5).

The Ca^{2+} homeostasis of 170 calcium stone-forming patients was studied on a self-chosen (random) diet, followed by 1 week of a low-Ca^{2+} diet, or after a 1 g oral Ca^{2+} load at the end of a 12 h fasting period (Table 3). When derived and heterozygous patients were compared, no differences were found with respect to plasma 1,25-dihydroxyvitamin D, PTH and Ca^{2+} (Table 3). Urinary Ca^{2+} excretion was also not affected under the above-mentioned dietary conditions (Table 3). The homozygous patient had a 3-fold higher Ca^{2+} excretion rate (1.32 mmol/mmol creatinine) after a 1 g oral Ca^{2+} load compared to derived and heterozygous patients (0.47 ± 0.27, 0.47 ± 0.28 mmol/mmol creatinine, respectively) (Table 3). The plasma PTH level was lower (24 pg/ml) in the homozygous patient compared to derived and heterozygous patients (43 ± 22, 42 ± 12 pg/ml, respectively). With free-choice and low Ca^{2+} diets, the Ca^{2+} excretion was 2-fold higher in the homozygous patient. This indicates that the patient had hypercalciuria under normal nutritional conditions. No differences in bone mineral density were found between groups (Table 3).

In the cohort of 170 Ca^{2+} stone formers, the frequency of the ancestral haplotype was higher [7.6% (derived = 314; ancestral = 26)] compared to 341 non-stone formers [5.4% (derived = 645, ancestral = 37] (Table 4). Similarly, the frequency of the ancestral haplotype was higher in 104 stone formers not investigated by the various calcium regimens (Table 4). The prevalence of the ancestral haplotype in stone-forming patients when compared to non-stone-forming patients (Table 4) was statistically significant (χ^2 test $P = 0.039$).

Data of three patients with the four non-synonymous polymorphisms (R138C+T269M, L259Q and A566P) are shown in Supplementary Material, Table S2. These patients were not included in the clinical-data analysis.

Functional analysis of TRPV6 polymorphisms

To investigate the functional significance of the three non-synonymous polymorphisms (157R+378V+681T), $^{45}Ca^{2+}$ transport assays were performed in *Xenopus* oocytes expressing the corresponding construct. The results indicated that Ca^{2+} uptake activity was higher in the ancestral TRPV6 when compared with derived TRPV6 ($n = 5$, $^{**}P < 0.01$, Fig. 1C). To estimate Ca^{2+} transport activity in heterozygous

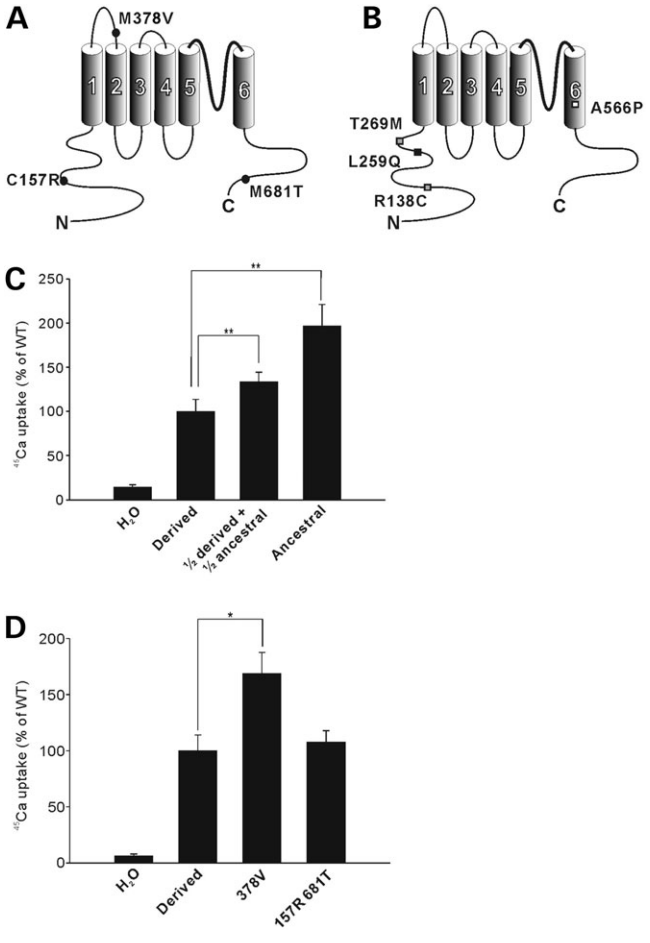

Figure 1. Functional analysis of the TRPV6 polymorphisms from Ca^{2+} stone patients. (**A**) Localization of three major non-synonymous TRPV6 polymorphisms in the TRPV6 protein. These three polymorphisms (C157R, M378V and M681T) were on the same allele/haplotype. (**B**) Two less frequent polymorphisms (R138C and T269M) were found in the same patient (*grey squares*). L259Q (*black square*) and A566P (*white square*) were low incidence single mutations/polymorphisms. (**C**) $^{45}Ca^{2+}$ uptake analysis in derived (C+M+M) or the ancestral (R+V+T) TRPV6 in *Xenopus* oocytes. The uptake of the ancestral TRPV6 was significantly higher (7.11 ± 1.93 pmol/min/oocyte, $n = 5$) compared to derived TRPV6 (3.61 ± 1.01 pmol/min/oocyte, $n = 5$). The co-expression of derived and ancestral TRPV6 resulted in an intermediate transport activity ($n = 8$), $^{**}P < 0.01$. (**D**) $^{45}Ca^{2+}$ uptake analysis with TRPV6s (derived TRPV6, 378V and 157R+681T). The 378V also significantly increased this activity ($n = 4$), $^{*}P < 0.03$.

patients, both derived and ancestral TRPV6 were co-injected into oocytes. The results showed intermediate activity for the combination of derived and ancestral TRPV6 (Fig. 1C). While the 157R+681T construct did not significantly increase Ca^{2+} transport activity ($P = 0.67$), the single extracellular polymorphism (378V) significantly increased Ca^{2+} transport activity ($n = 4$, $P < 0.03$, Fig. 1D), suggesting that 378V mainly contributes to the increase in Ca^{2+} transport activity of TRPV6.

DISCUSSION

In the present study, we found a haplotype consisting of three non-synonymous polymorphisms (C157R, M378V and M681T) in the TRPV6 gene in Ca^{2+} stone patients (Table 1). The frequency of the ancestral haplotype (R+V+T) was higher in Ca^{2+} stone formers (8.4%) when compared to a cohort of non-stone formers (5.4%). This suggests that the ancestral haplotype is a risk factor for Ca^{2+} stone formation (Table 3).

Although there was no difference in the phenotypes between heterozygous (CR+MV+MT) and derived (CC+MM+MM) patients, one ancestral patient was found who

Table 2. Baseline characteristics and clinical data

	CC+MM+MM ($n = 142$)	CR+MV+MT ($n = 24$)	RR+VV+TT ($n = 1$)
Male/female	111/31	17/7	1/0
Age (years)	44.3 ± 12.7	45.6 ± 16.0	45
Height (cm)	171 ± 13.1	173 ± 9	165
Weight (kg)	77.6 ± 16.8	74.5 ± 15.8	71
BMI (kg/m^2)	27.1 ± 13.0	24.7 ± 4.2	26.1
Family history of stones, *n*	51	10	No
Stone episodes per patient, *n*	2.9 ± 1.4	3.1 ± 1.4	>5
Stone type, *n*			
Calcium-oxalate	67	11	1
Calcium-phosphate	5		
Nephrocalcinosis	1		
Radio-opaque concrement	69	13	

Table 3. Clinical data

	CC+MM+MM ($n = 142$)	CR+MV+MT ($n = 24$)	RR+VV+TT ($n = 1$)
Plasma parameters			
Free-choice diet			
Ionized calcium (mmol/l)	1.23 ± 0.05	1.22 ± 0.03	1.22
Phosphate (mmol/l)	1.06 ± 0.18	1.07 ± 0.16	1.21
PTH (pg/ml)	43 ± 22	42 ± 12	24
1,25-vitamin D (pmol/ml)	123 ± 44	122 ± 59	Missing
Low calcium diet			
Ionized calcium (mmol/l)	1.20 ± 0.04	1.19 ± 0.03	1.19
Phosphate (mmol/l)	0.95 ± 0.17	0.97 ± 0.16	1.03
PTH (pg/ml)	45 ± 18	44 ± 13	27
1,25-vitamin D (pmol/ml)	146 ± 48	148 ± 65	188
Calciuria at/after			
Free-choice diet (mmol/day)	6.8 ± 3.7	6.9 ± 2.7	12.8
Low calcium diet (mmol/day)	4.0 ± 2.5	4.0 ± 2.0	8.2
Fasting, mmol/mmol creatinine	0.21 ± 0.14	0.18 ± 0.10	0.31
Calcium load, mmol/mmol creatinine	0.47 ± 0.27	0.47 ± 0.28	1.32
DEXA, z-score			
Lumbar spine	0.01 ± 0.94	0.26 ± 1.06	0.2
Femoral neck	0.32 ± 1.03	0.37 ± 0.94	0.5
Tibia diaphysis	0.61 ± 1.12	0.48 ± 1.03	1
Tibia epiphysis	−0.25 ± 0.96	−0.23 ± 0.94	−0.4

Table 4. Prevalence of the ancestral (RVT-) haplotype in stone-forming and non-stone-forming cohorts

	Number of subjects	CMM haplotype	RVT haplotype
Stone formers with metabolic workup	170	314 (92.4%)	26 (7.6%)
Stone formers without metabolic workup	104	188 (90.4%)	20 (9.6%)
Total stone formers	274	502 (91.6%)	46 (8.4%)*
Hypertensive	180	342 (95.0%)	18 (5.0%)
Healthy	161	303 (94.1%)	19 (5.9%)
Total non-stone formers	341	645 (94.6%)	37 (5.4%)*

*$P = 0.039$ (χ^2 test).

likely exhibited absorptive hypercalciuria. Indeed, when ancestral TRPV6 was expressed in *Xenopus* oocytes, $^{45}Ca^{2+}$ uptake activity was significantly higher compared to derived TRPV6 (Fig. 1C). This result suggests that the ancestral haplotype can increase intestinal Ca^{2+} absorption, resulting in absorptive hypercalciuria. However, in this study, there is thus far only one homozygous patient who exhibited absorptive hypercalciuria with kidney stones. In order to evaluate the phenotype–genotype relationship in more detail, additional homozygous patients with this haplotype need to be identified and analyzed. To determine whether the increase in Ca^{2+} transport activity is related to increased TRPV6 function, increased surface expression of TRPV6 or decreased protein degradation will be the subject of future investigations.

To address the effect of heterozygosity with respect to this haplotype, derived and ancestral TRPV6 were co-expressed into the same oocyte and $^{45}Ca^{2+}$ uptake was measured (Fig. 1C). The total $^{45}Ca^{2+}$ activity appeared to be the sum of the derived and ancestral TRPV6 activities, suggesting that the function of these channels was not affected by co-expression. It is generally assumed that four TRPV6 subunits form one channel pore (20). However, our results suggest that ancestral TRPV6 does not increase the activity of derived TRPV6 by forming heteromeric channels. This seems reasonable because there was no dominant effect of the ancestral haplotype in the Ca^{2+} stone patients (i.e. there was no difference in the phenotypes between derived and heterozygotes; see Table 2). Nevertheless, it is tempting to speculate that the heterozygous haplotype is still significant for the Ca^{2+} stone phenotypes. A combination of this haplotype with polymorphisms in other genes affecting Ca^{2+}-transport (i.e. TRPV5; ref. 21) may enhance the risk of stone formations because this disease is known to be a polygenic disease (1).

During the course of our study, the same ancestral haplotype (C157R+M378V+M681T) was found in a whole genome screening effort, and was reported to represent a positive selection in human evolution (14,22). In these reports, the derived haplotype accelerated TRPV6 protein evolution only in individuals of non-Southern African descent, suggesting that TRPV6 protein experienced geographically restricted selection pressure. The investigators hypothesized that this selection was caused either by a pathogen affecting the function of TRPV6-expressing B-cells or by the agricultural revolution involving the domestication of milk-producing animals $\sim$10 000 years ago. Too much Ca^{2+} from milk might be a strong selection pressure because it could cause absorptive hypercalciuria and urinary Ca^{2+} stones. Another possibility is that selection pressure is closely correlated with skin-color change and 1,25-dihydroxyvitamin D production (23,24). After moving from Africa to Europe, dark-skin individuals might need more ultraviolet radiation to produce 1,25-dihydroxyvitamin D. Even now, vitamin D deficiency is a common problem in individuals with darker skin pigmentation living in northern countries (25). Under this selection pressure, skin-color changes may have occurred by evolving several genes involved in pigmentation (i.e. tyrosinase, MC1R, SLC24A5 and SLC45A2) (26–28). Individuals with lighter skin pigmentation might then have produced too much 1,25-dihydroxyvitamin D, resulting in an increased intestinal Ca^{2+} absorption. Thus, to reduce the risk of absorptive hypercalciuria with kidney stones, the derived haplotype would have spread only among individuals with lighter skin pigmentation.

MATERIALS AND METHODS

Patients and clinical evaluation protocol

All patients ($n = 170$, no African individuals, Table 3) were seen for an outpatient metabolic work-up in our renal stone clinic between March 2004 and March 2007. Inclusion criteria were as follows: (i) age 18 plus of either gender, (ii) passage of at least one calcium-containing kidney stone as defined either by stone analysis or by the presence of opaque material on conventional radiograph or computed tomography in the absence of cystinuria. Exclusion criteria were: (i) an established cause of calcium stone formation, such as primary hyperparathyroidism, overt distal renal tubular acidosis, sarcoidosis, excessive vitamin D intake, hypercalciuria due to hypercalcemia (immobilization or malignancy) and primary or enteric hyperoxaluria (patients with inflammatory bowel disease and short bowel syndrome were excluded), (ii) creatinine clearance less than 60 ml/min as calculated from the serum and urinary creatinine of two 24 h urine collections and (iii) urinary tract infection. Patients were asked to stop calcium supplements or any drug that could affect the metabolism of calcium during the evaluation. All patients gave their consent for their participation and the protocol was approved by the institutional review board of the University Hospital Bern.

All patients underwent a three visit mineral metabolism work-up including a dual energy x-ray absorptiometry (DEXA). A routine clinical assessment was performed, including a physical examination and history. Mineral metabolism was investigated on four different calcium regimens according to a protocol first established by Pak *et al*. (15,16): (i) free-choice diet, (ii) low-calcium diet, (iii) fasting and (iv) after a 1 g oral calcium load.

In the 24-hour urines, calcium and creatinine were measured by standard laboratory techniques with an automated analyzer. Phosphate, calcium and creatinine in the blood were assessed by standard laboratory methods. Intact parathyroid hormone was measured by an enzyme-immunoassay (Roche Modular E170), as was 1,25-vitamin D (Immundiagnostik AG).

Hypercalciuria was defined as either fasting or absorptive. Fasting hypercalciuria was defined as an elevated fasting urinary calcium-to-creatinine ratio exceeding 0.31 mmol/mmol (0.11 mg/mg) in the 2 h urine sample. Absorptive hypercalciuria was defined as a urinary calcium-to-creatinine ratio of at least 0.56 mmol/mmol (0.20, mg/mg) in the 4 h urine sample after the oral intake of 1 g calcium (17). Osteodensitometry was performed by dual-energy X-ray absorptiometry at the second to fourth lumbar vertebra (DXA, Hologic® scanner QDR 1000 W).

To evaluate the ancestral haplotype in different patient cohorts, exon 3 was sequenced as a screening procedure in 104 additional stone-forming patients not previously investigated by applying four different calcium regimens, and also in 180 hypertensive patients and 161 non-hypertensive, healthy control subjects without a stone history (Table 3). Complete clinical data sets were not available from these patients.

Identification of TRPV6 polymorphisms. Genomic DNA was extracted from blood using QIAamp DNA Mini kit (Qiagen). For TRPV6 genomic analysis, primers for polymerase chain reactions (PCR) covering all coding regions of the exons and exon-intron boundaries were designed as listed in Supplementary Material, Table S1. PCR was performed using the AmpliTaq Gold system (Applied Biosystems) with 400 pmol of forward and reverse primers and 50 ng of genomic DNA. The condition for the PCR reactions was as follows: 35 cycles of denaturizing (94°C for 30 s), annealing [51°C (exon 9, 10), 52.5°C (exon 11, 12), 53.5°C (exon 7, 8), 54.5°C (exon 4), 55.5°C (exon1, 14), 57.5°C (exon 2, 3, 5, 6, 13, 15)] for 30 s, and extension (72°C for 1 min). After the final step at 72°C for 7 min, the fragment was confirmed by agarose gel electrophoresis as a single band. The sequence of this fragment was determined by ABI 3730XL Genetic Analyzer (Applied Biosystems) using Big Dye terminator cycle sequencing. When a polymorphism was detected, the sequence was read from both the forward and the reverse side. When the reference sequence (NCBI accession AY225461.2) of TRPV6 was detected, no additional reverse sequencing was performed.

Heterologous expression of human TRPV6 in Xenopus oocytes. All animal experiments were performed according to the Federal Guidelines of Switzerland. The oocyte expression vector containing human wild-type TRPV6 cDNA [Af365927; ref. 18] was constructed by NotI/ XhoI fragment of TRPV6 pBluescript. The constructs (derived TRPV6, 378V, 157*R*+681T and ancestral TRPV6) were generated using the QuikChange Multi site-directed mutagenesis kit (Stratagene), according to the manufacturer's instruction. A cRNA was synthesized *in vitro* by the mMessage mMachine kit (Ambion) with a linearized vector and SP6 RNA polymerase. For oocyte preparation, an ovary was isolated from *Xenopus laevis* under 2-aminoethyl benzoate treatment, and then incubated with collagenase A (Roche). The isolated oocytes were then incubated overnight at 17°C with modified Barths' solution (19). Oocytes were injected with 25 ng of cRNA and incubated at 17°C for 2 days before functional analysis.

Calcium-45 uptake assays. Oocytes were injected with 50 nl of 50 mM EGTA, 30 min before uptake assay. Calcium uptake assays were performed with standard uptake solution (100 mM NaCl, 2 mM KCl, 1.9 mM $MgCl_2$, 0.2 mM $CaCl_2$, 4μCi·ml^{-1} ^{45}Ca; Amersham). After incubation for 20 min, the oocytes were rinsed with the uptake solution and solubilized with 5% SDS. The ^{45}Ca content was analyzed by liquid scintillation counting.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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REFERENCES

1. Pak, C.Y. (1979) Physiological basis for absorptive and renal hypercalciurias. *Am. J. Physiol.*, **237**, F415–F423.
2. Coe, F.L., Evan, A. and Worchester, E. (2005) Kidney stone disease. *J. Clin. Invest.*, **115**, 2598–2608.
3. Moe, O.W. (2006) Kidney stones: pathophysiology and medical management. *Lancet*, **367**, 333–344.
4. Rendina, D., Mossetti, G., Viceconti, R., Sorrentino, M., Castaldo, R., Manno, G., Guadagno, V., Strazzullo, P. and Nunziata, V. (2004) Association between vitamin D receptor gene polymorphisms and fasting idiopathic hypercalciuria in recurrent stone-forming patients. *Urology*, **64**, 833–838.
5. Karnauskas, A.J., van Leeuwen, J.P., van den Bemd, G.J., Kathpalia, P.P., DeLuca, H.F., Bushinsky, D.A. and Favus, M.J. (2005) Mechanism and function of high vitamin D receptor levels in genetic hypercalciuric stone-forming rats. *J. Bone Miner. Res.*, **20**, 447–454.
6. Vezzoli, G., Terranegra, A., Arcidiacono, T., Biasion, R., Coviello, D., Syren, M.L., Paloschi, V., Giannini, S., Mignogna, G., Rubinacci, A. *et al.* (2007) R990G polymorphism of calcium-sensing receptor does produce a gain-of-function and predispose to primary hypercalciuria. *Kidney Int.*, **71**, 1155–1162.
7. Hoenderop, J.G., Nilius, B. and Bindels, R.J. (2005) Calcium absorption across epithelia. *Physiol. Rev.*, **85**, 373–422.
8. Suzuki, Y., Landowski, C.P. and Hediger, M.A. (2007) Mechanisms and regulation of epithelial Ca^{2+} absorption in health and disease. *Ann. Rev. Physiol.*, **70**, (ROI; 10.1146/annurev.physiol.69.031905.161003).
9. Peng, J.B., Chen, X.Z., Berger, U.V., Vassilev, P.M., Tsukaguchi, H., Brown, E.M. and Hediger, M.A. (1999) Molecular cloning and characterization of a channel-like transporter mediating intestinal calcium absorption. *J. Biol. Chem.*, **274**, 22739–22746.
10. Hoenderop, J.G., van der Kemp, A.W., Hartog, A., van de Graaf, S.F., van Os, C.H., Willems, P.H. and Bindels, R.J. (1999) Molecular identification

of the apical Ca^{2+} channel in 1,25-dihydroxyvitamin D-3-responsive epithelia. *J. Biol. Chem.*, **274**, 8375–8378.
11. Hoenderop, J.G., van Leeuwen, J.P., van der Eerden, B.C., Kersten, F.F., van der Kemp, A.W., Merillat, A.M., Waarsing, J.H., Rossier, B.C., Vallon, V., Hummler, E. *et al.* (2003) Renal Ca^{2+} wasting, hyperabsorption, and reduced bone thickness in mice lacking TRPV5. *J. Clin. Invest.*, **112**, 1906–1914.
12. Müller, D., Hoenderop, J.G., Vennekens, R., Eggert, P., Harangi, F., Mehes, K., Garcia-Nieto, V., Claverie-Martin, F., van Os, C.H., Nilius, B. and Bindels, R.J. (2002) Epithelial Ca^{2+} channel (ECAC1) in autosomal dominant idiopathic hypercalciuria. *Nephrol. Dial. Transplant.*, **17**, 1614–1620.
13. Bianco, S.D., Peng, J.B., Takanaga, H., Suzuki, Y., Crescenzi, A., Kos, C.H., Zhuang, L., Freeman, M.R., Gouveia, C.H., Wu, J. *et al.* (2007) Marked disturbance of calcium homeostasis in mice with targeted disruption of the *Trpv*6 calcium channel gene. *J. Bone Miner. Res.*, **22**, 274–285.
14. Akey, J.M., Swanson, W.J., Madeoy, J., Eberle, M. and Shriver, M.D. (2006) TRPV6 exhibits unusual patterns of polymorphism and divergence in worldwide populations. *Hum. Mol. Genet.*, **15**, 2106–2113.
15. Pak, C.Y.C., Kaplan, R., Bone, H., Townsend, J. and Waters, O. (1975) A simple test for the diagnosis of absorptive, resorptive and renal hypercalciurias. *New Engl. J. Med.*, **292**, 497–500.
16. Pak, C.Y., Britton, F., Peterson, R., Ward, D., Northcutt, C., Breslau, N.A., McGuire, J., Sakhaee, K., Bush, S., Nicar, M. *et al.* (1980) Ambulatory evaluation of nephrolithiasis. Classification, clinical presentation and diagnostic criteria. *Am. J. Med.*, **69**, 19–30.
17. Levy, F.L., Adams-Huet, B. and Pak, C.Y.C. (1995) Ambulatory evaluation of nephrolithiasis: an update of a 1980 protocol. *Am. J. Med.*, **98**, 50–59.
18. Peng, J.B., Brown, E.M. and Hediger, M.A. (2001) Structural conservation of the genes encoding CaT1, CaT2, and related cation channels. *Genomics*, **76**, 99–109.
19. Romero, M.F., Kanai, Y., Gunshin, H. and Hediger, M.A. (1998) Expression cloning using Xenopus laevis oocytes. *Methods Enzymol.*, **296**, 17–52.
20. Erler, I., Hirnet, D., Wissenbach, U., Flockerzi, V. and Niemeyer, B.A. (2004) Ca^{2+}-selective transient receptor potential V channel architecture and function require a specific ankyrin repeat. *J. Biol. Chem.*, **279**, 34456–34463.
21. Hoenderop, J.G., Voets, T., Hoefs, S., Weidema, F., Prenen, J., Nilius, B. and Bindels, R.J. (2003) Homo- and heterotetrameric architecture of the epithelial Ca^{2+} channels TRPV5 and TRPV6. *EMBO J.*, **22**, 776–785.
22. Biswas, S. and Akey, J.M. (2006) Genomic insights into positive selection. *Trends Genet.*, **22**, 437–446.
23. Rana, B.K., Hewett-Emmett, D., Jin, L., Chang, B.H., Sambuughin, N., Lin, M., Watkins, S., Bamshad, M., Jorde, L.B., Ramsay, M. *et al.* (1999) High polymorphism at the human melanocortin 1 receptor locus. *Genetics*, **151**, 1547–1557.
24. Jablonski, N.G. and Chaplin, G. (2000) The evolution of human skin coloration. *J. Hum. Evol.*, **39**, 57–106.
25. van der Meer, I.M., Karamali, N.S., Boeke, A.J., Lips, P., Middelkoop, B.J., Verhoeven, I. and Wuister, J.D. (2006) High prevalence of vitamin D deficiency in pregnant non-western women in The Hague, Netherlands. *Am. J. Clin. Nutr.*, **84**, 350–353.
26. Lamason, R.L., Mohideen, M.A., Mest, J.R., Wong, A.C., Norton, H.L., Aros, M.C., Jurynec, M.J., Mao, X., Humphreville, V.R., Humbert, J.E. *et al.* (2005) SLC24A5, a putative cation exchanger, affects pigmentation in zebrafish and humans. *Science*, **310**, 1782–1786.
27. Soejima, M., Tachida, H., Ishida, T., Sano, A. and Koda, Y. (2006) Evidence for recent positive selection at the human *AIM1* locus in a European population. *Mol. Biol. Evol.*, **23**, 179–188.
28. Norton, H.L., Kittles, R.A., Parra, E., McKeigue, P., Mao, X., Cheng, K., Canfield, V.A., Bradley, D.G., McEvoy, B. and Shriver, M.D. (2007) Genetic evidence for the convergent evolution of light skin in European and East Asians. *Mol. Biol. Evol.*, **24**, 710–722.