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TRPV5 gene polymorphisms in renal hypercalciuria

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Abstract

Background. Kidney stone formation is a major socioeconomic problem in humans, involving pain, recurrent treatment and renal insufficiency. As most renal precipitates contain calcium as a major component, hypercalciuria is the main risk factor for renal stone formation. Different forms of hypercalciuria can be classified, which primarily arise from defects in the main organs involved in calcium homeostasis. A distinction can be made between renal, absorptive and resorptive hypercalciuria, originating from disturbed calcium handling in kidney, intestine and bone, respectively. A positive family history predisposes individuals to an increased risk of stone formation, which strongly indicates the involvement of genetic susceptibility factors. TRPV5 is the renal epithelial calcium channel that is the gatekeeper protein in active calcium reabsorption in the kidney. TRPV5 gene ablation in mice leads to severe hypercalciuria, implying that TRPV5 is an interesting candidate gene for renal hypercalciuria in humans. This study aims to identify and functionally characterize TRPV5 gene aberrations in patients with renal hypercalciuria.

Methods. The TRPV5 coding region and intron–exon boundaries were screened for gene mutations in 20 subjects displaying renal hypercalciuria after which identified non-synonymous polymorphisms were functionally characterized by patch-clamp analysis. Wild-type and TRPV5 channels including polymorphisms were transiently expressed in human embryonic kidney (HEK) 293 cells and functionally characterized by patch-clamp analysis.

Results. Genotyping TRPV5 in renal hypercalciuria patients revealed three non-synonymous and five synonymous polymorphisms. Electrophysiological characterization of the TRPV5 mutants did not reveal significant functional changes compared to wild-type TRPV5 channel recordings.

Conclusions. In this specific patient cohort, our data do not support a primary role for TRPV5 in the pathogenesis of renal hypercalciuria. However, TRPV5 cannot be excluded as a candidate gene in hypercalciuria.

Keywords: calcium; hypercalciuria; ion channel; polymorphism; TRPV5

Introduction

Hypercalciuria is the main risk factor in renal calcium stone formation and constitutes universally an immense health and socioeconomic problem [1,2]. In the United States, ~5% of women and 12% of males develop a clinical episode of kidney stone disease during their lifetime [3], accompanied by severe pain and recurrent treatment. Approximately 80% of the renal stones contain calcium [4]. Besides hypercalciuria, other risk factors are low urinary volume, dietary components, gender and previous development of calcium stones [5,6]. Moreover, individuals with a positive family history of nephrolithiasis have an enhanced risk of experiencing kidney stones in their lifetime compared to those without a positive history [7]. This strongly implicates genetic factors as being involved in the pathogenesis of hypercalciuria-related renal stone formation.

The identification of a causal factor and the establishment of a categorization system for hypercalciuria remain difficult. Currently, a tripartite classification is being used, distinguishing renal, absorptive and resorptive hypercalciuria, of which the first two forms are the most common ones [8]. In the body, the concerted action of kidney, intestine and bone maintains a normal calcium balance. These organs constitute the sites where the above-mentioned forms of hypercalciuria primarily originate. As many transport proteins and hormones are involved in the maintenance of the calcium balance, defects in calcium homeostasis may have many causal factors. Therefore, hypercalciuria is a complex trait that can be polygenic and multifactorial in its aetiology. In the past, several candidate genes were identified and associated with hypercalciuria [9,10]. Others were unsuccessful in identifying loci or genetic variants linked to hypercalciuria, probably due to its heterogeneity [9,11–13]. Thus, the nature of the genetic defects involved in most cases of familial hypercalciuria remains fairly unknown.

Transient receptor potential vanilloid member 5 (TRPV5) constitutes the rate-limiting calcium entry pathway in renal transcellular calcium transport. The gene encoding the human TRPV5 channel is mapped to chromosome 7q35 and consists of 15 exons [14]. TRPV5 encodes a protein of 729 amino acids [14,15]. Interestingly, TRPV5

Table 1. Human TRPV5 primer sequences

Exon	Forward primer	Reverse primer
1	5'-TGCATACACAAGTCATACAGG-3'	5'-AGGTGGGTCAGAGGGTCTGAGG-3'
2-3	5'-TCATTCTGCTATGACTGTCTCTCC-3'	5'-ACACCCTCCATCTCAAAGTTTCC-3'
4-5	5'-ATCAGGAACCTCCTGAGAGGGATGG-3'	5'-ATGGGCCTCTGGCTTAATTAAGCC-3'
6-7	5'-TCAGCCTAGAACTGAGATATGGGG-3'	5'-ACCTCTATTCTCCCAGGGCCAGC-3'
8	5'-TAGATGCAGAGGTTTGGTGGTCC-3'	5'-AGGCCTGATCCTCCTTGGCATCC-3'
9	5'-ACTGATGAGCTGAGTGAGTGATGG-3'	5'-TCCCTGAGTAGCATGGCTTCG-3'
10-11	5'-ACCCACAATGGCAAGTACAATGG-3'	5'-AAGTCCTTGGAGTGAGAGTGACC-3'
12-13	5'-TACCACCCTGGTCACTACTATGC-3'	5'-AAGAATGAGAGTGAACCTTTCTGG-3'
14	5'-AGAATAAGTGCTAACCATGTCC-3'	5'-ATGATCCACCATAACATTTTCC-3'
15	5'-TGAGTGAAGCTGGTTGCATGGTGC-3'	5'-AGACAGGCTAATGCAGGCTCCAGG-3'

Oligonucleotide primers were developed for the amplification of each single exon of TRPV5, based on the genomic sequences. All primer sequences were located in non-coding regions to analyse intron–exon boundaries as well.

gene ablation in mice evokes a robust renal calcium leak, resulting in severe hypercalciuria. Besides hypercalciuria, the TRPV5 knockout mice demonstrate bone degradation, decreased bone mineral density, hypervitaminosis D, calcium hyperabsorption, normocalcaemia, polyuria and increased urinary acidification [16,17]. Regarding the crucial importance of TRPV5 in body calcium homeostasis and furthermore the renal calcium wasting observed in the knockout mice, TRPV5 is an important candidate gene for renal hypercalciuria.

The aim of our study was to investigate TRPV5 as a candidate gene for renal hypercalciuria. To this end, 20 patients were selected for hypercalciuria with concomitant polyuria or a low urinary pH and included in this study. These patients were screened for TRPV5 gene aberrations. The TRPV5 coding sequence and all exon–intron boundaries within the TRPV5 gene were analysed. Non-synonymous polymorphisms detected in TRPV5 were functionally characterized by patch-clamp analysis in human embryonic kidney (HEK) 293 cells.

Subjects and methods

Patient selection

Among the patients with idiopathic hypercalciuria, calcium nephrolithiasis or low bone mineral density attending the Department of Physiology of Georges Pompidou Hospital, Paris, France, a familial evaluation is commonly proposed. In this retrospective study, we screened the families in which at least two family members had idiopathic hypercalciuria and selected patients on the basis of the rate of urinary calcium excretion, the 24-h urine output volume and fasting urinary pH. A selection of 20 renal hypercalciuria patients was made. Patients with hypercalcaemia, hyperthyroidism or malignant neoplasm were not included. This study was performed in agreement with the bio-ethic French law. Informed consent was obtained from all patients participating in the study.

Clinical evaluation

Subjects were evaluated after 2 days on a calcium (400 mg/24 h) and sodium (150 mmol/24 h) diet, preceded by a 2-week period without antiacid, non-steroidal anti-inflammatory drugs, diuretic agents, glucocorticoids, calcium, phosphate or vitamin supplements. A 24-h urine sample was collected during the last day of the diet to measure total calcium excretion. A 1-h fasting urine sample was obtained in order to measure urinary pH, calcium and creatinine concentrations. Fasting blood samples were obtained and analysed for plasma total and serum ionized calcium, parathyroid hormone (PTH), 1,25-dihydroxyvitamin D, 25-hydroxyvitamin D₃ and creatinine levels.

Serum and urine biochemistry

Total urinary and plasma calcium concentrations were measured by atomic absorption spectrometry (Perkin Elmer 3300, Norwalk, CT, USA). Serum ionized calcium levels were determined by an ICA2 electrode (Copenhagen, Denmark). Sodium levels were assessed by flame spectrophotometry (Instruments Laboratory, Lexington, MA, USA). Urinary and serum phosphate levels were analysed with a colorimetric reaction on a Technicon RA-XT chemistry analyser (Bayer, Germany). Creatinine levels were measured according to Jaffé's method (RA-XT). Intact (1–84) serum PTH was determined by the Nichols Allegro PTH immunoradiometric assay (Nichols Institute, San Juan Capistrano, CA, USA). Serum 25-hydroxyvitamin D₃ levels were obtained by a radioimmunoassay, whereas 1,25-dihydroxyvitamin D concentration measurements were performed with a radioreceptor assay (Incstar, Stillwater, MN, USA). Urinary pH values were determined with an electronic ion analyser.

Phenotype assignment

Hypercalciuria was defined as a 24-h urinary calcium excretion exceeding 0.1 mmol/kg body weight on a non-restricted diet. Renal hypercalciuria was defined by a ratio of fasting urinary calcium/creatinine (fasting UCa/UCr) > 0.37 [18].

DNA extraction

DNA sample collection and storage were carried out according to standard methods. In short, 10 ml of peripheral blood was collected into EDTA-containing tubes at the time of initial diagnosis and centrifuged for 10 min at 3000 g to separate buffy coats and plasma. Total genomic DNA was isolated from the buffy coat using a QIAmp Blood Kit (Qiagen S.A., Courtaboeuf, France) according to the blood and body fluid protocol recommended by the manufacturer. Extracted DNA was quantified by spectrophotometrical absorbance measurements.

Genotyping

Oligonucleotide primers were developed for the amplification of each single exon of TRPV5, based on the genomic sequence. All primer sets obtained from Biolegio (Nijmegen, The Netherlands) that were used to amplify the genomic DNA of TRPV5 by polymerase chain reaction (PCR) are depicted in Table 1. Because of short intron sequences between exons 2 and 3, 4 and 5, 6 and 7, 10 and 11 as well as 12 and 13 of TRPV5, these exons were amplified and sequenced as single fragments. Primer sequences were located in non-coding regions to analyse the intron–exon boundaries as well. PCR product purifications of all exons were performed with the GenElute™ PCR Clean-up Kit (Sigma, St Louis, MO, USA) or Qiaex® II Gel extraction kit (Qiagen, Hilden, Germany). DNA sequencing was carried out by standard procedures at the sequence facility of the Radboud University Nijmegen Medical Centre. Subsequently, we assessed the alignment and comparison of the patient sequences with the human TRPV5 mRNA sequence, obtained from the NCBI nucleotide database (NM_019841).

DNA constructs

To investigate the channel function of the TRPV5-A8V, R154H and A561T gene polymorphisms, the mutations were each prepared by *in vitro* mutagenesis [Quickchange[®] (Stratagene)] and cloned into the homologous wild-type rabbit TRPV5 cDNA in the pCINeo/IRES-GFP vector [19,20]. The wild-type TRPV5 and TRPV5 mutant constructs were verified by cDNA sequence analyses.

Electrophysiological analysis

The full-length cDNA encoding wild-type or mutated TRPV5 was transfected into HEK293 cells, as described previously [19,20]. Patch-clamp experiments were performed in the tight seal whole-cell configuration at room temperature using an EPC-9 patch-clamp amplifier controlled by the Pulse software (HEKA Elektronik, Lambrecht, Germany). Pipettes had resistances between 2 and 5 m Ω when filled with the recording solution. Capacitance and series resistance were compensated. Access resistance was monitored continuously using the automatic capacitance compensation of the Pulse software. Sodium currents were measured using a ramp protocol. Cells were held at +20 mV and voltage ramps of 450-ms duration each, ranging from -100 to +100 mV, were applied every 5 s. Extracellular solution for sodium currents contained in mM 150 NaCl, 6 CsCl, 10 glucose and 10 HEPES/CsOH (pH 7.4). Calcium currents were studied using a 3-s voltage step to -100 mV from a holding potential of +70 mV. NaCl, 150 mM, was replaced with an equimolar amount of N-methyl-D-glucamine-Cl (NMDG-Cl) from extracellular solution and 10 mM calcium was added to measure the calcium current. Current densities were obtained by normalizing the current amplitude to the cell membrane capacitance. Data were analysed using Igor pro software (Wavemetrics, Lake, Oswego, NY, USA).

Statistical analyses

Electrophysiological data analysis was performed using IgorPro software (Wavemetrics, Lake, Oswego, NY, USA). Data were depicted as means \pm SEMs. Significance was determined using Student's *t*-test with *P* < 0.05 considered to be statistically significant.

Results

Patient selection and biochemical analyses

In order to investigate the involvement of TRPV5 gene aberrations in hypercalciuria, 20 subjects were included in this study. All subjects demonstrated renal hypercalciuria (mean fasting UCa/UCr 0.48 ± 0.02), with concomitant polyuria or low urinary pH (Table 2). The mean urinary sodium excretion was 140 mmol/24 h (range: 40–300 mmol/24 h). All patients showed normal serum PTH and calcium levels. Mean 25-hydroxyvitamin D₃ level was 42 ± 5 nM, whereas the serum 1,25-dihydroxyvitamin D concentration was 110 ± 6 pM.

Sequence analysis in renal hypercalciuria patients

Polymorphisms identified in the coding sequence of TRPV5 in renal hypercalciuria patients and expression rates are listed in Table 3. The topology of the TRPV5 protein is depicted in Figure 1. Detected synonymous polymorphisms were L205L, Y222Y, Y278Y, T281T and T344T. The non-synonymous gene aberrations identified were A8V, R154H and A561T. Both the A8V (dbSNP rs# cluster id: rs4252372) and R154H (rs4236480) polymorphisms were previously identified and are listed in single nucleotide polymorphism (SNPs) databases (<http://www.ncbi.nlm.nih.gov/SNP/>). Allele frequency data in the European control population showed 96% C- and

Table 2. Blood and urine biochemistry of included subjects

Blood	Subjects	Normal
Total calcium (mM)	2.26 ± 0.02	2.10–2.55
Ionized calcium (mM)	1.23 ± 0.01	1.15–1.32
Creatinine (μ M)	75 ± 3	70–125
PTH (pg/ml)	38 ± 5	11–57
1,25(OH) ₂ D (pM)	110 ± 6	45–110
25(OH)D ₃ (nM)	42 ± 5	20–100
Urine		
Urinary calcium excretion (mmol/24 h)	8.5 ± 0.4	<8.0
Urinary volume (l/24 h)	2.4 ± 0.1	1.0–1.5
Fasting UCa/UCr (mmol/mmol)	0.48 ± 0.02	<0.4
Fasting urinary pH	6.0 ± 0.1	>5.0
Age (years)	44 ± 3	
Weight (kg)	69 ± 3	

A selection of 20 renal hypercalciuria patients was made. Renal hypercalciuria was defined by a ratio of fasting urinary calcium/creatinine (fasting UCa/UCr) > 0.37 mmol/mmol. Data are expressed as means \pm SEMs.

Table 3. TRPV5 gene polymorphisms detected in renal hypercalciuria patients

Exon	Polymorphism	Ht patients (%)	Hm patients (%)
1	A8V	20	0
2			
3			
4	R154H	55	10
5			
6	L205L	40	0
7		50	5
8		40	0
9		50	5
10	T281T	45	5
11			
12	A561T	10	0
13			
14			
15			

TRPV5 allele expression rates are depicted as percentage of patients (*n* = 20) demonstrating the specific polymorphism in a heterozygous (Ht) and homozygous (Hm) manner.

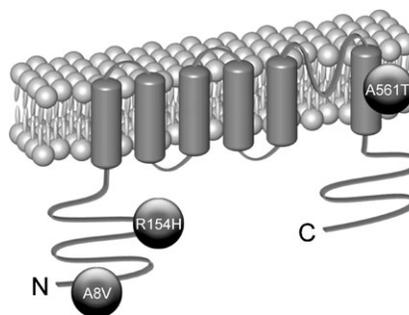


Fig. 1. Gene polymorphisms in the TRPV5 channel. The TRPV5 protein topology is depicted in a putative 3-dimensional model, displaying six transmembrane regions and intracellular N- and C-tails. Depicted in black are the three TRPV5 non-synonymous polymorphisms demonstrated in renal hypercalciuria patients, which were functionally characterized by patch-clamp analysis.

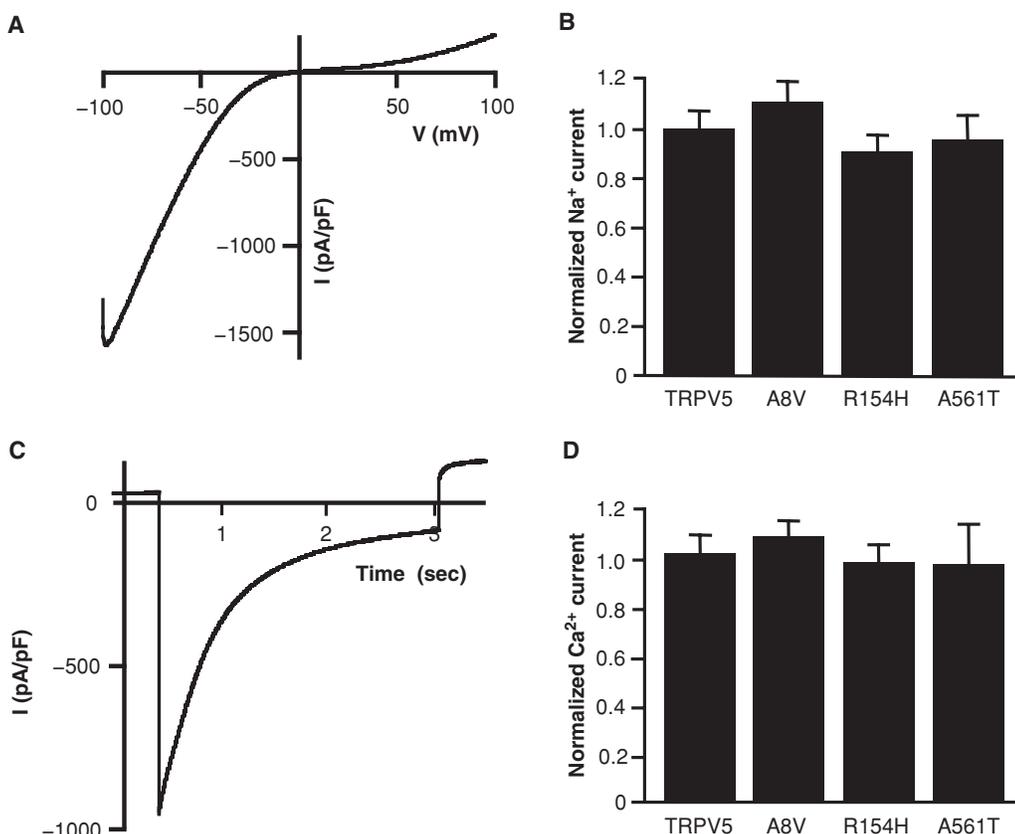


Fig. 2. Electrophysiological analysis of wild-type and mutant TRPV5 channels. Sodium (A and B) and calcium (C and D) currents in TRPV5 channels. (A) Current–voltage relations for wild-type TRPV5 recorded in a divalent-free solution. (B) Histogram plotting the peak values at -80 mV for sodium currents recorded from HEK293 cells transiently transfected with wild-type TRPV5 or mutant channels. (C) Calcium-induced inactivation of the calcium current was measured with 10 mM extracellular calcium during a 3-s step to -100 mV from a holding potential of $+70$ mV in cells over expressing wild-type TRPV5. (D) Histogram plotting the peak values for calcium currents at -100 mV recorded from HEK293 cells over expressing wild-type TRPV5 or TRPV5 mutant channels. Current amplitudes were normalized to the amplitude of wild-type TRPV5.

4% T-allele frequencies for A8V, whereas R154H showed 38% A- and 62% G-allele frequencies (NCBI ss# assay id: ss69030695). A561T frequencies were not determined in the general population so far.

Effect of non-synonymous polymorphism on TRPV5 channel activity

The TRPV5 non-synonymous polymorphisms were characterized by electrophysiological analysis. To this end, each construct (wild-type TRPV5 and mutant TRPV5-A8V, TRPV5-R154H and TRPV5-A561T) was transiently transfected into human embryonic kidney (HEK293) cells and the current characteristics were analysed by patch-clamp measurements (Figure 2). HEK293 cells expressing wild-type TRPV5 displayed the typical inwardly rectifying current–voltage relation in a divalent-free solution (Figure 2A, 1137 ± 83 pA/pF at -80 mV, $n = 14$) and a calcium-dependent inactivation of the calcium current (Figure 2C, 938 ± 86 pA/pF at -100 mV, $n = 13$). As shown in Figure 2B and D, the normalized amplitudes of both sodium and calcium peak currents were not significantly different between wild-type TRPV5 and mutant channels.

In order to investigate whether the calcium-dependent inactivation pattern of TRPV5 was changed in the mutant proteins, a 3-s voltage step to -100 mV from a holding potential of $+70$ mV was applied to the cells. No significant difference was recorded between wild-type TRPV5 and mutant TRPV5 calcium-dependent inactivation.

Discussion

A positive family history forms a risk in the development of hypercalciuria. Genes encoding calcium transport proteins that are involved in the maintenance of the body calcium balance are candidate genes in the pathogenesis of hypercalciuria. Interestingly, mice genetically ablated for the renal epithelial calcium channel TRPV5 demonstrate a severe renal calcium leak. In the present study, TRPV5 gene aberrations were analysed and identified in 20 renal hypercalciuria patients with at least one affected relative. All subjects displayed concomitant polyuria or decreased urinary pH, which are symptoms presented by TRPV5 knockout mice as well. Synonymous as well as non-synonymous polymorphisms were detected in the TRPV5 coding sequences of renal hypercalciuria patients. Electrophysiological analysis

of the TRPV5-A8V, R154H and A561T mutants compared to wild-type TRPV5 did not show significant changes in channel function. In this specific research population, our data do not support a primary role for TRPV5 in the pathogenesis of renal hypercalciuria. TRPV5 can, however, not be excluded as a candidate gene in hypercalciuria.

An increased risk for the development of hypercalciuria is present when a first-degree relative is diagnosed with hypercalciuria [7]. This fact is a strong indication for the existence of genetic susceptibility factors in the aetiology of hypercalciuria. The epithelial calcium channel TRPV5, which is a key player in renal active calcium reabsorption was previously postulated to be an important candidate gene in calcium metabolism-associated disorders [21]. Interestingly, characterization of the TRPV5 knockout mouse model revealed that a functional defect of this channel causes a severe renal calcium leak [16,22]. Muller *et al.* previously screened for TRPV5 gene mutations in nine families known to have autosomal-dominant hypercalciuria [23]. No TRPV5 mutations were demonstrated. In the present study, 20 subjects were selected for renal hypercalciuria and concomitant polyuria or increased urinary acidification in order to obtain a homogeneous patient cohort. Although matched for symptoms presented by the TRPV5 knockout animal model, no disease-causing TRPV5 gene aberrations were detected, as revealed from functional, electrophysiological analyses [23].

Non-synonymous gene polymorphisms lead to amino acid changes that can alter the function of a protein. Therefore, we determined the electrophysiological properties of mutated TRPV5 channels containing non-synonymous polymorphisms detected in the renal hypercalciuria patients. Patch-clamp analysis showed no significant changes in sodium and calcium peak currents or calcium-dependent inactivation curves in mutant TRPV5 proteins compared to wild-type TRPV5. This finding suggests that the individual A8V, R154H and A561T polymorphisms do not play a key role in the aetiology of hypercalciuria in this studied patient cohort. TRPV5 cannot be excluded though, as a candidate gene for renal hypercalciuria. The heterogeneity of hypercalciuria and the potential contribution of several calcium transport systems in this complex trait are enormous. Therefore, gene association studies should be preferentially initiated in large, well-characterized patient groups. In this perspective, specific haplotypes of several polymorphisms together could cause an increased susceptibility to hypercalciuria. Previously, unusual SNPs patterns and haplotype differences were identified in the intestinal calcium channel TRPV6, a homologue of TRPV5 involved in the absorption of calcium in the intestine, among worldwide populations [24]. Akey *et al.* suggested that a specific TRPV6 haplotype comprising three non-synonymous SNPs (C157R, M378V and M681T) resulted in a selective advantage during human history. This might indicate a different TRPV6 channel function related to specific haplotypes. A recent study, investigating the functional characterization of these TRPV6 variants by patch-clamp analysis, revealed no significant differences in biophysical channel function, although calcium-dependent inactivation might be affected in the presence of specific TRPV6 haplotypes [25]. Unfortunately, the polygenic nature and the uncertainties of an

exact diagnosis for hypercalciuria hamper population-based association studies for genetic hypercalciuria. In addition, linkage analysis is a powerful method to identify genes involved in familial hypercalciuria. Previously, Reed *et al.* performed a genome-wide search in three families with absorptive hypercalciuria. A high maximum logarithm of the odds score >12 was found for the region 1q23.4 to 24 in which a hypothetical human orthologue of the rat soluble adenylate cyclase was identified [26,27]. So far, a causative relationship between soluble adenylate cyclase and hypercalciuria has not been identified. The latter investigations underline the importance of further efforts to study monogenic causes of hypercalciuria.

In summary, TRPV5 gene polymorphisms were identified in renal hypercalciuria patients that were well characterized and selected for concomitant polyuria or decreased urinary pH, symptoms presented by the TRPV5 knockout mouse model. Electrophysiological analyses of a non-synonymous TRPV5 gene aberration did not implicate the A561T polymorphism in the aetiology of renal hypercalciuria. TRPV5 cannot be excluded as a candidate gene for hypercalciuria, as TRPV5 gene variants might be involved in other hypercalciuric patient cohorts. Due to the heterogeneity of hypercalciuria and the possible involvement of multiple calcium transport systems in this complex trait, gene association studies should be performed in large, well-characterized patient groups displaying hypercalciuria.

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Conflict of interest statement. None declared.

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