Frequency of Rare Allelic Variation in Candidate Genes among Individuals with Low and High Urinary Calcium Excretion

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

Citation
Toka, Hakan R., Giulio Genovese, David B. Mount, Martin R. Pollak, and Gary C. Curhan. 2013. "Frequency of Rare Allelic Variation in Candidate Genes among Individuals with Low and High Urinary Calcium Excretion." PLoS ONE 8 (8): e71885. doi:10.1371/journal.pone.0071885. http://dx.doi.org/10.1371/journal.pone.0071885.

Published Version
doi:10.1371/journal.pone.0071885

Citable link
http://nrs.harvard.edu/urn-3:HUL.InstRepos:11855873

Terms of Use
This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA
Frequency of Rare Allelic Variation in Candidate Genes among Individuals with Low and High Urinary Calcium Excretion

Hakan R. Toka1,2, Giulio Genovese2, David B. Mount1, Martin R. Pollak2, Gary C. Curhan1,3
1 Division of Nephrology, Brigham and Women’s Hospital, Boston, Massachusetts, United States of America, 2 Division of Nephrology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts, United States of America, 3 Channing Division of Network Medicine, Boston, Massachusetts, United States of America

Abstract

Our study investigated the association of rare allelic variants with extremes of 24-hour urinary calcium excretion because higher urinary calcium excretion is a dominant risk factor for calcium-based kidney stone formation. We resequenced 40 candidate genes potentially related to urinary calcium excretion in individuals from the Nurses’ Health Studies I & II and the Health Professionals Follow-up Study. A total of 960 participants were selected based on availability of 24-hour urine collection data and level of urinary calcium excretion (low vs. high). We utilized DNA sample pooling, droplet-based target gene enrichment, multiplexing, and high-throughput sequencing. Approximately 64% of samples (n = 615) showed both successful target enrichment and sequencing data with >20-fold deep coverage. A total of 259 novel allelic variants were identified. None of the rare gene variants (allele frequencies <2%) were found with increased frequency in the low vs. high urinary calcium groups; most of these variants were only observed in single individuals. Unadjusted analysis of variants with allele frequencies ≥2% suggested an association of the Claudin14 SNP rs113831133 with lower urinary calcium excretion (6/520 versus 29/710 haplotypes, P value = 0.003). Our data, together with previous human and animal studies, suggest a possible role for Claudin14 in urinary calcium excretion. Genetic validation studies in larger sample sets will be necessary to confirm our findings for rs113831133. In the tested set of candidate genes, rare allelic variants do not appear to contribute significantly to differences in urinary calcium excretion between individuals.

Introduction

Kidney stone disease is a major cause of morbidity associated with tremendous pain, suffering, and substantial economic impact [1–3]. The majority of kidney stones contain calcium, most commonly in the form of calcium (Ca2+) oxalate. Higher urinary Ca2+ excretion is associated with higher risk of calcium-containing kidney stone formation. The etiology of the vast majority of cases of hypercalciuria is unknown and is referred to as idiopathic hypercalciuria [4].

Nephrolithiasis is a multifactorial disease with genetic and environmental factors determining the likelihood of stone formation [5]. We and other investigators have identified multiple environmental risk factors associated with increased risk including lower dietary Ca2+ intake [6–8], lower fluid intake [6–9], and higher body mass index [8,10]. A family history of nephrolithiasis is associated with a greater than two-fold increase in the risk of developing a stone [11]. Substantial data demonstrate that calcium-based kidney stones and elevated urinary Ca2+ are linked and likely have a strong genetic component [12]. Genes for rare Mendelian forms of nephrolithiasis and increased urinary Ca2+ excretion have been identified; however, mutations in these genes explain only a very small fraction of kidney stone disease in the general population. For example, mutations in the renal chloride channel CLCN5 cause Dent’s disease, a group of X-linked hypercalciuric abnormalities [13]. Inactivating (loss-of-function) mutations in the calcium-sensing receptor (CaSR) cause autosomal-dominant hypocalcemia and hypercalcuiuria [14]. Another example is familial hypomagnesemia associated with hypercalcuiuria and nephrocalcinosis (mutations in Claudin16) [15]. Additional genes were identified in various animal models such as the renal epithelial Ca2+ transporter gene (TRPV5), which when mutated causes severe hypercalcuiuria in the mouse [16].

Rather than testing the “common disease, common variant” hypothesis pursued by genome wide association studies (GWAS), our approach tested the association of the frequencies of rare genetic variants with 24-hour urinary Ca2+ excretion [17]. This approach has succeeded when examining other complex traits such as hypertriglyceridemia [18], hypercholesterolemia [19], and non-alcoholic fatty liver disease [20]. The findings in these studies were consistent with in silico predictions that some sequence variations found in healthy individuals are as deleterious to protein function as mutations that, in other genes, cause monogenic disease. Highly penetrant rare alleles may be an important genetic
contributor to common disease seen in the general population as shown for blood pressure variation [21].

The goal of this work was to identify rare, functionally significant genetic variants associated with urinary Ca$^{2+}$ excretion. Forty candidate genes possibly related to urinary Ca$^{2+}$ excretion were resequenced at extremes of urinary Ca$^{2+}$ excretion in 960 individuals from three well-characterized cohorts, the Nurses’ Health Studies (NHS) I & II and the Health Professional Follow-Up Study (HPFS).

Methods

A. Study cohorts

The NHS I was established in 1976 with over 120,000 female registered nurses aged 30–55 years. The NHS II was established in 1989 with over 116,000 female nurses aged 25–42 years. The HPFS was established in 1986 with over 51,000 male health care professionals aged 40–75 years. All three cohorts have been followed by biennially mailed questionnaires including questions on lifestyle practices and newly diagnosed diseases such as nephrolithiasis [22]. Additional information was obtained from self-reported cases including symptoms and kidney stone type. In validation studies, permission to obtain medical records was requested from newly diagnosed cases in all three cohorts. The diagnosis of stone disease was confirmed in over 90% of these cases. Twenty-four-hour urine collections were obtained from participants with a history of confirmed nephrolithiasis and from randomly selected controls. Those with a history of kidney stones performed the collections after the diagnosis. All 24-hour urine collections were performed using the Mission Pharmacal system (San Antonio, TX, USA). Urinary Ca$^{2+}$ was measured by an atomic absorption spectrophotometer [22]. Approximately 10% of individuals with the highest and lowest values of 24-hour urinary Ca$^{2+}$ excretion from available male and female participants in equal numbers were selected.

This study was approved by the Brigham and Women’s Hospital’s institutional review board (approval # 2000P00131). The institutional review board (IRB) specifically considered the risks and anticipated benefits, if any, to participants, and the selection, safety and privacy of individuals. Implied consent was considered as appropriate by the IRB for this specific study.

B. DNA samples

DNA samples were collected as part of a general collection of blood samples in the three cohort studies. We limited this study to those who self-reported their race as Caucasian (and this was confirmed as part of a separate GWAS analysis). High quality DNA was extracted (Dana Farber/Harvard Cancer Center) from buffy coats via Qamp 96 spin-protocol (Qiagen Inc., Chatsworth, CA). DNA concentrations were calculated in 96-well format using a Molecular Dynamics spectrophotometer. The 960 DNA samples were ranked by urinary Ca$^{2+}$ excretion and grouped into pools of 15 or 20 samples prior to target DNA capture. A pilot project testing pooled samples of 15 versus 20 individual samples did not show any differences in DNA capture efficiency and sequence coverage per individual samples between the pools (data not shown). We subjected 32 sample pools (N = 960 individuals; 16 pools of 15 samples and 36 pools of 20 samples) to RainDance target (RDT) DNA capture (Table 1). The amount of genomic DNA provided for RDT capture per pool was 10 μg (0.5–0.66 μg per individual sample).

C. Candidate genes

Candidate genes were selected by searching public databases (PubMed and Online Mendelian Inheritance in Man (OMIM)). We limited the number of genes to 40 due to cost and technical limitations at the time of study design. Our priority was to achieve sufficient sequence coverage (at least 20x) to detect rare allelic variation. Selection of candidate genes was based on in vivo and in vitro evidence of regulating Ca$^{2+}$ homeostasis in bone, kidney or intestine. We also selected some genes, such as oxalate and citrate exchangers, which may affect calcium-based stone formation by changing supersaturation rather than urinary Ca$^{2+}$ excretion per se. The genes resequenced in this study are listed in Table 2, including gene name, gene/protein function, RefSeq ID and exon number. References underlining the rationale of gene selection are provided. As an additional gene, we included PIK3C2G (phosphoinositide-3-kinase, class 3, gamma polypeptide) based on our unpublished GWAS data. PIK3C2G regulates diverse cellular responses, such as cell proliferation, oncogenic transformation, cell migration, intracellular protein trafficking, and cell survival.

D. Primer design

Our list of the 40 genes was provided to RainDance Technologies (RDT), (Lexington, MA) for custom primer design based on the Primer3 algorithm (http://frodo.wi.mit.edu/primer3). The custom panel was prepared and primers were designed to target all 497 exons of the 40 candidate genes, including ~50 bp of intronic sequence flanking each exon. The 795 amplicons in the panel ranged in size from 200 to 600 bases, with a GC content of 25% to 87%, and represented a total coding sequence of ~182 kb. All single nucleotide polymorphisms (SNPs) and repeat regions were filtered from the primer selection region. The RDT design was quality checked to ensure that none of the primers were designed over known SNPs and primer sequences were verified to avoid repetitive regions of the genome using the program RepeatMasker (http://www.repeatmasker.org). The primers for the 795 amplicons varied in annealing temperature from 57°C to 60°C, with a primer length range of 15 to 22 bases. Other rules for primer design included BLASTing the primers to the chromosome that contained the gene of interest and in silico PCR to match the designed primers to PCR product and target sequence.

E. Enrichment of target DNA for sequencing

The capture was performed at two laboratories, RDT in Lexington, MA, and Ambry Genetics in Aliso Viejo, CA. DNA samples were fragmented to 3 to 4 kb by shearing the genomic DNA with the Covaris S2 instrument (Covaris, Woburn, MA) following the manufacturer’s instructions. To prepare the input DNA template mixture for targeted amplification, 3 μg of the purified genomic DNA fragments were added to 4.7 μL of high-fidelity buffer (Invitrogen, Carlsbad, CA), 1.26 μL of magnesium sulfate (Invitrogen), 1.6 μL of 10 mmol/L dNTP (Invitrogen), 3.6 μL of 4 mol/L betaine (Sigma-Aldrich, St. Louis, MO), 3.6 μL of Droplet Stabilizer (RDT, Lexington, MA), 1.8 μL of dimethyl sulfoxide (Sigma-Aldrich), and 0.7 μL of Platinum High-Fidelity Taq (Invitrogen). The samples were brought to a final volume of 25 μL with nuclease-free water. PCR droplets were generated on the RDT1000 instrument. The enrichment panel consisted of an emulsion that contained a collection of unique primer droplets in which each primer droplet contained a single matched forward and reverse primer for each amplicon in the panel. Each panel contained multiple replicates of each unique primer droplet with consistent volume. The RDT1000 generated a PCR droplet by pairing a single genomic DNA template droplet.
with a single primer droplet. The paired droplets flowed past an electrode in the RDT chip and were instantly merged to create a single PCR droplet. All of the resulting PCR droplets were dispensed as an emulsion into a PCR tube and then transferred to a standard thermal PCR cycler for amplification (GeneAmp 9700 thermocycler, Applied Biosystems, Foster City, CA). After PCR amplification, the emulsion was broken to release each individual amplicon from the PCR droplets. For each sample, an equal volume of Droplet Destabilizer (RDT) was added to the emulsion of PCR droplets, the sample was vortexed for 15 sec, and spun in a microcentrifuge at 13,000 × g for 5 min. The oil below the aqueous phase was carefully removed from the sample and the remaining sample was purified using a MinElute column (Qiagen, Valencia, CA) following the manufacturer’s recommended protocol. The purified amplicon DNA was tested on an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA) to confirm that it matches the expected amplicon profile (mixture of amplicons ranging from 200 to 600 bp in size).

F. Targeted deep DNA sequencing

A simplified schematic illustration of our emulsion-based droplet PCR approach and the off-chip work flow before high-throughput sequencing is presented in Figure 1. Successfully enriched sample pools were barcoded and sequenced on the Hiseq2000 Illumina platform (7 barcoded sample pools per lane). After PCR purification, amplified fragments for each individual were repaired to blunt ends using NEB Quick blunting kit (NEB, catalog # E1201L, 15 min RT). The PCR fragments were then linked using NEB Quick ligation kit (NEB, catalog # M2200L). Ligation was done overnight at 25°C. The ligated products were made into 100 μL volume by adding elution buffer and were then sheared using Covaris E210 (Covaris, Woburn, MA). The sheared fragments were purified using Qiagen QIAquick PCR purification column and eluted in 32 μL of elution buffer. The samples then entered the standard Illumina Genome Analyzer multiplex library introduced preparation protocol. The enrichment was confirmed by running an Agilent BioAnalyzer 7500 DNA chip. A quantitative PCR was done to quantitate the library using KAPA Library quantification kit (KAPA Biosystems, Woburn, MA, USA, catalog # KK4824). Enriched DNA was denatured and diluted to a concentration of 8 pM. Seventy bp single end sequencing was performed using standard IGAII manuals and version 4 kits. Seven sample pools per lane of Illumina sequencing were multiplexed. After sequencing, the reads consisting of 795 fragments covering 262,545 bases were mapped and variants sites identified against the reference sequence using the BWA software [23]. This region is larger than the actual targeted bases (182,345) as RDT included some intronic and intergenic regions to facilitate primer picking. All sequence data aligned for the analysis had sequence coverage exceeding 20-fold. Sequences were compared to those reported by HapMap using a custom perl script to assess the rates of data completion and accuracy. The HapMap data was assumed to be without error when estimating data accuracy.

G. Variant identification

Utilizing the Syzygy software [24], we compared all identified single nucleotide variants (SNVs) with three publicly available SNP databases including dbSNP (http://www.ncbi.nlm.nih.gov/projects/snp/), 1000 Genome Project (1000G) [25], and Exome Sequencing Project (ESP) (http://evs.gs.washington.edu/ESV/) databases. After filtering known SNPs, we used the transition to transversion ratio (Ti/Tv) to filter out false positive novel variants, which occur frequently on high-throughput sequencing platforms. Ti/Tv can be used to estimate true-positive (TP) to false-positive (FP) SNP data [26]. A transition is the mutation of a purine nucleotide to another purine (A<>G) or a pyrimidine to another pyrimidine (C<>T). In contrast, a transversion (Tv) substitutes a purine for a pyrimidine or vice versa. The initially observed Ti/Tv ratio in our SNV data was a mixture of true-positive and false-positive SNVs. This was based on the assumption that if all detected SNVs are true, Ti/Tv equals ~3.3. If all SNVs are false Ti/Tv equals 0.5. We assumed that common novel variants were most likely artifacts of sequencing. We therefore considered novel variants only if they were observed less than or equal to 3 times in the whole dataset. In nature, Tis are more common than Tvs. We used the following equation to estimate the number of true positive SNVs: %TP = (Ti/Tvobs-Ti/TvFP)/(Ti/TvTP-Ti/TvFP). Considering that Ti/TvTP is around 2.8–3.3, the %TP should be ~9–11%.

Results

In order to query the potential relevance of rare allelic variation in genes associated with urinary Ca2⁺ excretion, we decided to amplify and sequence the exons of 40 genes in 960 well-characterized individuals from the NHS and HPFS populations as outlined in the methods section (see Tables 1 and 2, Figure 1) Efficacy of DNA enrichment and sequencing

Forty-two out of 52 samples pools were successfully captured (N = 730 individual samples). Ten sample pools (N = 230) failed enrichment due to technical issues. The 42 samples pools were barcoded and further pooled (in sets of 7 sample pools) for sequencing on the HiSeq2000 Illumina platform. Eight samples pools had insufficient sequence coverage (<2-fold). Thirty four sample pools (N = 615) showed sequencing data with >20-fold sequence coverage. These raw sequence data were deposited into the NIH short read archive (SRA) database (http://www.ncbi.
nlm.nih.gov/sra) under the accession number PRJNA209216. The target DNA showed uniform amplification across all target amplicons.

The distribution of study participants with 20-fold sequence coverage from the three cohorts in low (N = 355) and high (n = 260) urinary calcium excretion groups are shown in Table 3. The phenotype data provided include urinary solute excretion, age and body mass index (BMI). The number of kidney stone formers was significantly higher in the high urinary Ca²⁺ group (n = 164 vs. n = 96; Chi Square P value = 0.004).

### Allelic variation in targeted DNA sequence

Samples pools that passed our quality matrix, by enriching for target DNA and showing over 20-fold sequence coverage, were included in our analysis (N = 615 individuals). The total number of identified sequence nucleotide variants (SNVs) with Szygy software was 1,572 (Figure 2A). Of these, 429 were known SNPs based on

### Table 2. Candidate genes included in the resequencing study (n = 40).

| Gene | Gene name/protein function | RefSeq ID | Exons |
|------|---------------------------|-----------|-------|
| Aconitase [32] | Catalyzes the isomerization of citrate to isocitrate | NM_001098 | 18 |
| CaSR [33] | Calcium-sensing receptor | NM_000388 | 7 |
| Citrate lyase [34] | Catalyzes the formation of acetyl-CoA from citrate | NM_001096 | 29 |
| Claudin 2 [35] | Tight junction protein, proximal tube (PT) | NM_001171092 | 2 |
| Claudin 8 [35] | Tight junction protein, primarily distal nephron (DCT) | NM_99328 | 1 |
| Claudin 10 [36] | Tight junction protein, thick ascending limb (TAL) and intestine | NM_001160100 | 5 |
| Claudin 14 [37] | Tight junction protein, TAL | NM_001146077 | 3 |
| Claudin 16 [15] | Tight junction protein, TAL | NM_006580 | 4 |
| Claudin 19 [30] | Tight junction protein, TAL and DCT | NM_001123395 | 5 |
| CLCN5 [38] | Chloride channel 5, mutations cause Dent’s disease | NM_000804 | 15 |
| CLCNKA [39] | Basolateral chloride channel expressed in TAL | NM_001042704 | 20 |
| CLCNKB [40] | Basolateral chloride channel expressed in TAL and DCT; mutation cause type III Bartter’s syndrome | NM_000085 | 20 |
| FGF23 [41] | Fibroblast growth factor 23, phosphatonin | NM_020638 | 3 |
| GCMB [42] | Glial cell missing B, mutations cause familial isolated hypoparathyroidism | NM_004752 | 5 |
| Klotho [43] | Regulator of TRPVS and FGF23 | NM_004795 | 5 |
| NHERF1 [44] | Hydrogen exchanger regulatory factor 1, mutations cause hypophosphatemia and nephrolithiasis | NM_004252 | 6 |
| NHERF2 [45] | Hydrogen exchanger regulatory factor 2, expressed like NHERF1 in PT | NM_00130012 | 7 |
| NKCC2 [46] | Na⁺-K⁺-2Cl⁻ :cotransporter, mutations cause type 1 (neonatal) Bartter’s syndrome | NM_000220 | 27 |
| PDZK1 [47] | Hydrogen exchanger regulatory factor 3, PT | NM_002614 | 10 |
| PIK3C2G | Phosphoinositide-3-kinase, class 3, γ polypeptide | NM_004570 | 32 |
| PTH [48] | Parathyroid hormone | NM_000315 | 3 |
| PTHR [49] | Parathyroid hormone receptor 1 | NM_000316 | 16 |
| ROMK [50] | Renal outer medullary K⁺ channel, mutations cause type 2 Bartter’s syndrome | NM_000338 | 4 |
| SLC12A3 [51] | Thiolase-sensitive Na⁺-Cl⁻ :cotransporter, mutation cause Gitelman’s syndrome | NM_000339 | 26 |
| SLC13A2 [52] | Na⁺ citrate transporter NaC1 | NM_001145975 | 12 |
| SLC13A3 [53] | Na⁺ citrate transporter NaC2 | NM_001193340 | 14 |
| SLC25A1 [54] | Mitochondrial citrate transporter | NM_005984 | 9 |
| SLC26A1 [55] | Oxalate and sulfate anion transporter | NM_004425 | 4 |
| SLC26A2 [56] | Oxalate and citrate exchanger | NM_000112 | 2 |
| SLC26A6 [57] | Oxalate and citrate exchanger | NM_01040454 | 21 |
| SLC34A1 [58] | Na⁺ phosphate co-transporter NaPi2A | NM_003052 | 13 |
| SLC34A3 [59] | Na⁺ phosphate co-transporter NaPi2C | NM_00177316 | 13 |
| SLC4A1 [60] | AE1 oxalate, mutations cause distal RTA | NM_000342 | 20 |
| SLC4A2 [61] | AE2 oxalate | NM_003040 | 23 |
| SLC4A3 [61] | AE3 oxalate | NM_005070 | 23 |
| TRPV5 [62] | Epithelial Ca²⁺ channel ECaC1 | NM_019841 | 14 |
| TRPV6 [63] | Epithelial Ca²⁺ channel ECaC2 | NM_018646 | 15 |
| UMOD [64] | Uromodulin | NM_001008389 | 11 |
| VDR [65] | Vitamin D receptor | NM_000376 | 11 |
| WNK4 [66] | Protein kinase, lysine deficient 4, mutations cause pseudohypoaldosteronism type 2 | NM_032387 | 19 |

doi:10.1371/journal.pone.0071885.t002

Role of Allelic Variation in Calcium Excretion
The frequency of kidney stone formers was significantly higher (*) in the high urinary Ca\textsuperscript{2+} excretion group (Chi Square P value = 0.004). The common, synonymous CLDN14 SNP rs219780, previously shown to be associated with kidney stone disease in a large GWAS study [27], did not differ between the two urinary Ca\textsuperscript{2+} groups (unadjusted P value = 0.82).

**Discussion**

The goal of this work was to quantify the frequency of rare, presumably functional genetic variants in individuals with lower and higher urinary Ca\textsuperscript{2+} excretion because urinary Ca\textsuperscript{2+} is a major risk factor for calcium-based kidney stone disease [4]. A different approach has been pursued previously in a large GWAS for kidney stone disease and has shown only limited success possibly due to the complex mechanistic nature of nephrolithiasis. That GWAS in 3,773 kidney stone cases and 42,510 controls from Iceland and the Netherlands reported an association of CLDN14 with nephrolithiasis. The synonymous CLDN14 variant rs219780(C) (minor allele frequency ~20%) showed a significant association with kidney stone disease (P value = 4\times10^{-12}) and low bone mineral density (for the hip P value = 0.00039) [27].

In this study, we tested the association of the frequencies of rare allelic variants with possible effect on urinary Ca\textsuperscript{2+} excretion by resequencing 40 known genes that could potentially affect or correlate with urinary Ca\textsuperscript{2+} excretion either in human disease or animal models. Our main hypothesis was that differing number of rare variants between individuals with lower and higher urinary Ca\textsuperscript{2+} excretion would be identified in at least some of these candidate genes. Rare, non-synonymous variants in low and high urinary Ca\textsuperscript{2+} excretion groups could contribute to the level of urinary Ca\textsuperscript{2+} excretion (lowering or increasing excretion and thereby protecting from or predisposing to calcium-based stone formation). This approach has succeeded for other phenotypes such as hyperlipidemia [18],[19] and non-alcoholic fatty liver disease [20], where the frequency of rare gene variants was significantly different in the extremes of the studied phenotype. The results in those studies were consistent with in silico predictions that rare amino acid changing sequence variations found in healthy individuals are as deleterious to protein function as gene mutations causing Mendelian disease. Such sequence variations may explain a significant fraction of phenotypic variation in human as suggested for blood pressure variation in the Framingham Heart Study population [21].

We identified 1,572 single nucleotide variants (SNVs), most of which appeared common in our subjects. Initially, we filtered out known gene variants based on three large SNP databases and then applied a simple but very efficient method the Ti/Tv of naturally occurring mutations [26]. We filtered potentially false-positive SNVs, significantly reducing the initially seen number of SNVs and improving the Ti/Tv from 0.75 to a “normal” range of 2.88.
Of the 259 novel SNPs in 40 different genes, none showed a significantly increased frequency in the low versus the high urinary Ca\textsuperscript{2+} excretion groups. These extremely rare candidate gene variants identified mostly in single individuals could be functionally contributing to the level of urinary Ca\textsuperscript{2+} excretion, because they are mostly amino acid changing and occur frequently only in one of the two groups with extreme urinary Ca\textsuperscript{2+} excretion. Identification of the individuals carrying these very rare variants and testing their relatives (for degree of urinary Ca\textsuperscript{2+} excretion and presence of variant) would help to answer if these variants contribute to the phenotype. Examining the conservation of the affected residue across species as well as the \textit{in vitro} and \textit{in vivo} effects on gene function would be necessary to postulate a cause-effect relation for these very rare variants.

We also analyzed our data for rare variants with allele frequency of 2–5%. Of these, the non-synonymous CLDN14 SNP rs113831133 (minor allele frequency for \(~\sim 3.3\%\) for c.11C>T, p.Thr4Met, dbSNP) showed a lower allele frequency in individuals with high urinary Ca\textsuperscript{2+} excretion (\(~\sim 1.1\%\) vs. \(~\sim 4.1\%\) in the lower urinary Ca\textsuperscript{2+} group), suggesting that if present it may lower urinary Ca\textsuperscript{2+} excretion, probably by decrease in CLDN14 function. The functional significance of the CLDN14 SNP rs113831133 in the thick ascending limb is unknown. Since heterozygous CLDN14-deficient mice have no renal phenotype, a dominant negative effect of rs113831133 appears more likely than haploinsufficiency [28]. The rs113831133 finding is in particular interesting since the synonymous CLDN14 SNP rs219780 has been implicated previously in kidney stone disease [27]. The frequency of this SNP was not significantly different in our study groups, though our study included a smaller sample size and our focus was on urine Ca\textsuperscript{2+} excretion rather than kidney stone formation. There are several different studies implicating an

### Table 4. Distribution of all identified Claudin14 SNPs in the low and high urinary Ca\textsuperscript{2+} excretion groups with unadjusted Chi square P values.

| Chr. Position | SNP ID (rs…) | SNP class | Nucleotide | Low urine Ca\textsuperscript{2+} | High urine Ca\textsuperscript{2+} | P       |
|---------------|--------------|-----------|------------|-------------------------------|-------------------------------|---------|
| 37833330      | .            | missense  | c.664G>T   | 2.2% (16/710)                | 1.5% (8/520)                 | 0.41    |
| 37833331      | .            | silent    | c.663G>A   | 0.0% (0/710)                 | 0.2% (1/520)                 | -       |
| 37833934      | .            | silent    | c.60C>T    | 0.1% (1/710)                 | 0.2% (1/520)                 | -       |
| 37833931      | 117560775    | silent    | c.63G>A    | 1.4% (10/710)                | 3.2% (17/520)                | 0.03    |
| 37833694      | 113350364    | silent    | c.300C>T   | 0.4% (3/710)                 | 0.0% (0/520)                 | -       |
| 37833699      | .            | missense  | c.295G>A   | 0.0% (0/710)                 | 0.3% (1/520)                 | -       |
| 37833751      | 219779       | silent    | c.243C>T   | 24.2% (172/710)              | 23.8% (124/520)              | 0.89    |
| 37833797      | .            | silent    | c.15C>T    | 0.0% (0/710)                 | 0.2% (1/520)                 | -       |
| 37833809      | .            | missense  | c.185A>G   | 0.4% (3/710)                 | 0.0% (0/520)                 | -       |
| 37833892      | .            | silent    | c.102G>A   | 0.1% (1/710)                 | 0.0% (0/520)                 | -       |
| 37833865      | .            | silent    | c.129C>T   | 0.3% (2/710)                 | 0.0% (0/520)                 | -       |
| 37833506      | .            | missense  | c.488C>T   | 0.3% (2/710)                 | 0.2% (1/520)                 | -       |
| 37833983      | 113831133    | missense  | c.11C>T    | 4.1% (29/710)                | 1.1% (6/520)                 | 0.003   |
| 37833976      | .            | silent    | c.18G>A    | 0.3% (2/710)                 | 0.0% (0/520)                 | -       |
| 37833661      | 74934405     | silent    | c.333A>C   | 4.1% (29/710)                | 4.4% (23/520)                | 0.77    |
| 37833304      | .            | silent    | c.690C>T   | 0.1% (1/710)                 | 0.1% (1/520)                 | -       |
| 37833307      | 219780       | silent    | c.687G>A   | 18.4% (131/710)              | 18.9% (98/520)               | 0.82    |

The missense variant rs113831133, highlighted in bold, was more frequent among individuals with lower urinary Ca\textsuperscript{2+} excretion. This association did not reach statistical significance when adjusted for multiple comparisons.

doi:10.1371/journal.pone.0071885.g002
important role for CLDN14 in urinary Ca\textsuperscript{2+} excretion. CLDN14 has been shown to be a negative regulator of the CLDN16/19 complex in the TAL, which has an important role in the paracellular Ca\textsuperscript{2+} reabsorption in the TAL [29]. Mutations in both CLDN16 and 19 have been shown to cause familial hypercalciuria in human. In addition, recent animal studies showed a significant role for CLDN14 in urinary Ca\textsuperscript{2+} reabsorption [29,30]. Homozygous CLDN14-deficient mice display lower urinary Ca\textsuperscript{2+} excretion than wild-type controls when challenged with a high calcium diet [28]. Renal tubule-specific Casr-deficient mice display decreased urinary Ca\textsuperscript{2+} excretion compared to control animals. CLDN14 is significantly downregulated (~80%) in this mouse model [31].

Our study had several limitations. The selection of candidate genes was biased and several other candidate genes potentially related to urinary Ca\textsuperscript{2+} excretion were not included in our investigation. Therefore, an unbiased approach including all genes would be most favorable. This could be accomplished by exome capture followed by next-generation sequencing including all coding regions of the genome. Another limitation is the relatively low sample number reducing the power to test the main hypothesis of this study. In order to further examine the role of rare, potentially functional variants, e.g. with minor allele frequency of 1% or lower, a larger sample group is needed. Our study was designed to include sequencing data of 960 individuals, however, due to technical difficulties we were only able to include 615 samples.

Conclusions

Our study does not support the hypothesis that rare, presumably functional allelic variants in the tested genes influence urinary Ca\textsuperscript{2+} excretion. Although an association of CLDN14 with urinary Ca\textsuperscript{2+} excretion was observed, this finding didn’t reach statistical significance. Yet, our data combined with the previous GWAS, recent human and animal data suggest a potential role for CLDN14 in urinary Ca\textsuperscript{2+} excretion. Further studies of CLDN14 are required to study its contribution to urinary Ca\textsuperscript{2+} excretion and calcium-based kidney stone disease.

Supporting Information

Table S1 Novel SNPs identified in low and high urinary Ca\textsuperscript{2+} excretion cohorts.

Author Contributions

Conceived and designed the experiments: HRT GG DBM MRP GCC. Performed the experiments: HRT GG. Analyzed the data: HRT GG DBM MRP GCC. Drafting the article or revising it critically for important intellectual content: HRT GG DBM MRP GCC. Contributed reagents/materials/analysis tools: HRT GG DBM MRP GCC. Table S1 Novel SNPs identified in low and high urinary Ca\textsuperscript{2+} excretion cohorts.

References

1. Pearle MS, Callhoun EA, Curhan GC (2005) Urologic diseases in America project: urolithiasis. J Urol 173: 848–857.
2. Yoshida O, Okada Y (1990) Epidemiology of urolithiasis in Japan: a chronological and geographical study. Urol Int 45: 104–111.
3. Stamatelou KK, Francis ME, Jones CA, Nyberg LM, Curhan GC (2003) Time trends in reported prevalence of kidney stones in the United States: 1976–1994. Kidney Int 63: 1817–1823.
4. Worceter EM, Coe FL (2010) Clinical practice. Calcium kidney stones. N Engl J Med 363: 954–965.
5. Attanasio M (2011) The genetic components of idiopathic nephrolithiasis. Pediatr Nephrol 26: 337–346.
6. Curhan GC, Willett WC, Rimm EB, Stampfer MJ (1995) A prospective study of dietary calcium and other nutrients and the risk of symptomatic kidney stones. N Engl J Med 333: 833–838.
7. Curhan GC, Willett WC, Speizer FE, Spiegelman D, Stampfer MJ (1997) Comparison of dietary calcium with supplemental calcium and other nutrients as factors affecting the risk for kidney stones in women. Ann Intern Med 126: 497–504.
8. Curhan GC, Willett WC, Knight EL, Stampfer MJ (2004) Dietary factors and the risk of incident kidney stones in younger women: Nurses' Health Study II. Arch Intern Med 164: 885–891.
9. Borioli I, Meschi T, Amato F, Briganti A, Novarini A, et al. (1996) Urinary volume, water and recurrences in idiopathic calcium nephrolithiasis: a 5-year randomized prospective study. J Urol 155: 839–843.
10. Taylor EN, Stamper MJ, Curhan GC (2003) Obesity, weight gain, and the risk of kidney stones. JAMA 290: 453–462.
11. Curhan GC, Willett WC, Rimm EB, Stampfer MJ (1997) Family history and risk of kidney stones. J Am Soc Nephrol 8: 1568–1573.
12. Moe OW, Bonny O (2005) Genetic hypercalciuria. J Am Soc Nephrol 16: 729–745.
13. Wrong OM, Norden AG, Feest TG (1994) Dent's disease: a familial proximal renal tubular syndrome with low-molecular-weight proteinuria, hypercalciuria, nephrocalcinosis, metabolic bone disease, progressive renal failure and a marked male predominance. QJM 87: 473–493.
14. Pollak MR, Brown EM, Estey HL, McLaine PN, Kifor O, et al. (1994) Autosomal dominant hypocalciuric hypercalcemia caused by a Ca(2+)sensing receptor gene mutation. Nat Genet 8: 303–307.
15. Simon DR, Lu Y, Chosat KA, Velazquez H, Al-Sabban E, et al. (1999) Paracellin-1, a renal tight junction protein required for paracellular Mg2+ resorption. Science 285: 103–106.
16. Hoenderop JG, van Leeuwen JP, van der Eerden BC, Kersten FF, van der Knaap JW, et al. (2003) Renal Ca2+ wasting, hyperabsorption, and reduced bone thickness in mice lacking TRPV5. J Clin Invest 112: 1906–1914.
17. Maher B (2008) Personal genomes: The case of the missing heritability. Nature 456: 18–21.
18. Romeo S, Pennacchio LA, Fu Y, Boersma E, Tybjaerg-Hansen A, et al. (2007) Population-based resequencing of ANGPTL4 uncovers variations that reduce triglycerides and increase HDL. Nat Genet 39: 513–516.
19. Fahimi S, Yang C, Esmail S, Hobs HH, Cohen JC (2008) Functional characterization of genetic variants in NPC1L1 supports the sequencing extremes strategy to identify complex trait genes. Hum Mol Genet 17: 2101–2107.
20. Romeo S, Koditima J, Xing C, Pertemudlidis A, Cox D, et al. (2008) Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. Nat Genet 40: 1461–1465.
21. JI W, Foo JN, O’Roak BJ, Zhao H, Larson MG, et al. (2008) Rare independent mutations in renal salt handling genes contribute to blood pressure variation. Nat Genet 40: 592–599.
22. Curhan GC, Taylor EN (2008) 24-h uric acid excretion and the risk of kidney stones. Kidney Int 73: 489–496.
23. Li H, Dubin R (2010) Fast and accurate long-read alignment with Burrows-Wheeler transform. Bioinformatics 26: 509–515.
24. Rivas MA, Beaudoin M, Gardet A, Stevens C, Sharma Y, et al. (2011) Deep resequencing of GWAS loci identifies independent rare variants associated with inflammatory bowel disease. Nat Genet 43: 1066–1073.
25. Consortium GP (2010) A map of human genome variation from population-scale sequencing. Nature 467: 1061–1073.
26. DePristo MA, Banks E, Poplin R, Garimella KV, Maguire JR, et al. (2011) A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nat Genet 43: 491–498.
27. Thorleifsson G, Holm H, Edwardsson V, Walters GB, Styrkkorslett D, et al. (2009) Sequence variants in the CLDN14 gene associate with kidney stones and bone mineral density. Nat Genet 41: 926–930.
28. Gong Y, Renigunta V, Himmerkus N, Zhang J, Renigunta A, et al. (2012) Claudin-14 regulates renal Ca2+(+) transport in response to CaSR signalling via a novel microRNA pathway. EMBO J 31: 399–2012.
29. Konrad M, Schaller A, Nedlow D, Panley AW, Waldegger S, et al. (2006) Mutations in the tight-junction gene Claudin 19 (CLDN19) are associated with renal magnesium wasting, renal failure, and severe ocular involvement. Am J Hum Genet 79: 949–957.
30. Hou J, Renigunta A, Gomes AS, Hou M, Paul DL, et al. (2009) Claudin-16 and Claudin-19 interaction is required for their assembly into tight junctions and for renal reabsorption of magnesium. Proc Natl Acad Sci U S A 106: 15350–15355.
31. Toku HR, Al-Romaili K, Koshy JM, Diharto S, Kos CH, et al. (2012) Renal tubule-specific calcium-sensing receptor deficient mice display PTH-independent hypocalciuria. J Am Soc Nephrol 54: 160–165.
33. Pearce SH, Williamson C, Kifor O, Bai M, Cosyhardt MG, et al. (1996) A familial syndrome of hypocalcemia with hypercalciuria due to mutations in the calcium-sensing receptor. N Engl J Med 335: 1115–1122.

34. Tooskhowong P, Tungaguna K, Phongudom S, Sriboonruang P (2005) Effects of potassium-magnesium citrate supplementation on cytosolic ATP citrate lyase and mitochondrial aconitase activity in leukocytes: a window on renal citrate metabolism. Int J Urol 12: 140–144.

35. Hou J, Rajagopal M, Yu AS (2013) Claudins and the kidney. Annu Rev Physiol 75: 479–501.

36. Breiderhof T, Himmerkus N, Stiiver M, Mütig K, Will C, et al. (2012) Deletion of claudin-10 (Cldn10) in the thick ascending limb impairs paracellular sodium permeability and leads to hypermagnesemia and nephrocalcinosis. Proc Natl Acad Sci U S A 109: 14241–14246.

37. Dimke H, Desai P, Borovac J, Lau A, Pan W, et al. (2013) Activation of the Ca(2+)-sensing receptor increases renal claudin-14 expression and urinary Ca(2+) excretion. Am J Physiol Renal Physiol 304: F761–769.

38. Lloyd SE, Pearce SH, Fisher SE, Steinmeyer K, Schwappach B, et al. (1996) A role of the Na-K-2Cl cotransporter in the familial syndrome of Bartter's syndrome. J Clin Invest 98: 267–273.

39. Uchida S (2000) Physiological role of CLC-K1 chloride channel in the kidney. Nephrol Dial Transplant 15 Suppl 6: 14–15.

40. Simon DB, Bindra RS, Mansfield TA, Nelson-Williams C, Mendonca E, et al. (1996) A glucocorticoid inducible kinase SGK1. Cell Physiol Biochem 15: 175–182.

41. Rendina D, Mossetti G, De Filippo G, Cioffi M, Strazzullo P (2006) Requirement of PDZ domains for the stimulation of the epithelial Ca2+ channel, ROMK. Nat Genet 1: 149–152.

42. Parkinson DB, Thakker RV (1992) A donor splice site mutation in the parathyroid hormone gene is associated with autosomal recessive hypoparathyroidism. J Med Genet 29: 299–312.

43. Yoshida T, Fujimori T, Nabeshima Y (2002) Mediation of unusually high concentrations of 1,25-dihydroxyvitamin D in homozygous idiotho mutant mice by increased expression of renal 1α-hydroxylase gene. Endocrinology 143: 603–609.

44. Kamin Z, Gerard B, Bakouh N, Alili R, Leroy C, et al. (2008) NHERF1 adaptors: new insights into cystic fibrosis, secretory diarrhoea and fructose-malabsorption. Cell Physiol Biochem 29: C1363–1375.

45. Palmada M, Poppendieck S, Embark HM, van de Graaf SF, Boehmer C, et al. (2010) Gain-of-function mutations and responsiveness of renal parathyroid hormone. N Engl J Med 359: 4022–4027.

46. Talhouk AS, Moroni P, Pinheiro-Souza L, Muflih N, Pittet B, et al. (2013) Association of variants at UMOD with chronic kidney disease and kidney stones-role of age and comorbid diseases. PLoS Genet 6: e1001039.

47. Seidler U, Singh A, Chen M, Cinar A, Bachmann O, et al. (2009) Knockout mouse models for intestinal electrolyte transporters and regulatory PDZ adaptors: new insights into cystic fibrosis, secretory diarrhoea and fructose-induced hypertension. Exp Physiol 94: 175–179.

48. Parkinson DB, Thakker RV (1992) A donor splice site mutation in the parathyroid hormone gene is associated with autosomal recessive hypoparathyroidism. Nat Genet 1: 149–152.

49. Ba J, Brown D, Friedman PA (2003) Calcium-sensing receptor regulation of PTH-inhibitable proximal tubule phosphate transport. Am J Physiol Renal Physiol 285: F1233–1243.

50. Simon DB, Karet FE, Rodriguez-Soriano J, Hamdan JH, DiPietro A, et al. (1996) Genetic heterogeneity of Bartter's syndrome revealed by mutations in the K+ channel, ROMK. Nat Genet 14: 152–156.

51. Simon DB, Nelson-Williams C, Bia MJ, Ellison D, Karet FE, et al. (1996) Gitelman's variant of Bartter's syndrome, inherited hypokalaemic alkalosis, is caused by mutations in the thiazide-sensitive Na-Cl cotransporter. Nat Genet 12: 24–30.

52. Okamoto N, Aruga S, Matsuzaki S, Takahashi S, Matsumita K, et al. (2007) Associations between renal sodium-citrate cotransporter (NaK2Cl) gene polymorphism and urinary citrate excretion in recurrent renal stone formers and normal controls. Int J Urol 14: 344–349.

53. Bergeron MJ, Clemenson B, Hedger MA, Markovich D (2013) SLC13 family of Na+-coupled di- and tricarboxylate/sulfate transporters. Mol Aspects Med 34: 299–312.

54. Nota R, Struyf EA, Pop A, Jansen EE, Fernandez Ojeda MR, et al. (2013) Deficiency in SLC25A1, encoding the mitochondrial citrate carrier, causes combined D-2- and L-2-hydroxyglutaric aciduria. Am J Hum Genet 92: 627–631.

55. Dawson PA, Russell CS, Lee S, McLeay SG, van Dongen JM, et al. (2010) Urilaterality and heterotopuria are linked to the anion transporter Sat1 in mice. J Clin Invest 120: 706–712.

56. Heneghan J, Akhavein A, Salas MJ, Shmulder BE, Karniski LP, et al. (2010) Regulated transport of sulfate and oxalate by SLC26A2/DITDST. Am J Physiol Cell Physiol 290: C1363–1375.

57. Wang Z, Wang T, Petrovic S, Tao B, Riederer B, et al. (2005) Renal and intestinal transport defects in Slc26a6-null mice. Am J Physiol Cell Physiol 288: C957–965.

58. Chau H, El-Mazdawy S, McKee MD, Tenenhouse HS (2003) Renal calcification in mice homozygous for the disrupted type Ia Na/Pi cotransporter gene Npt2. J Bone Miner Res 18: 644–637.

59. Ichikawa S, Sorensen AH, Insel EA, Friedman NE, Gertner JM, et al. (2006) Intronic deletions in the SLC34A3 gene cause hereditary hypophosphatemic rickets with hypercalcemia. J Clin Endocrinol Metab 91: 4022–4027.

60. Zhang Z, Liu XX, He JW, Fu WZ, Yue H, et al. (2012) Identification of two novel mutations in the SLC34A1 gene in two unrelated Chinese families with distal renal tubular acidosis. Arch Med Res 43: 299–304.

61. Alper SL, Darman RB, Chernova MN, Dahl NK (2002) The AE gene family of anion transporters and respiratory pathogens. N Engl J Med 349: 1128–1135.

62. Palamad M, Poppendieck S, Embark HM, van de Graaf SF, Boehmer C, et al. (2005) Requirement of PDZ domains for the stimulation of the epithelial Ca2+ channel TRPV5 by the NHE regulating factor NHERF2 and the serum and glucocorticoid inducible kinase SGK1. Cell Physiol Biochem 15: 175–182.

63. Suzuki Y, Pasch A, Bonny O, Mohaupt MG, Hediger MA, et al. (2008) Gain-of-function haplotype in the epithelial calcium channel TRPV6 is a risk factor for renal calcium stone formation. Hum Mol Genet 17: 1613–1618.

64. Gudbjartsson DF, Holm H, Indridason OS, Thorleifsson G, Edvardsson V, et al. (2010) Association variants at UMOD with chronic kidney disease and kidney stones-role of age and comorbid diseases. PLoS Genet 6: e1001039.

65. Guillaufjardt BF, Holm H, Indridason OS, Thorleifsson G, Edvardsson V, et al. (2010) Association of variants at UMOD with chronic kidney disease and kidney stones-role of age and comorbid diseases. PLoS Genet 6: e1001039.