Decreased Transcriptional Activity of *Calcium-sensing receptor* Gene Promoter 1 Is Associated With Calcium Nephrolithiasis

Giuseppe Vezzoli,* Annalisa Terranegra,* Andrea Aloia,* Teresa Arcidiacono, Luciano Milanesi, Ettore Mosca, Alessandra Mingione, Donatella Spotti, Daniele Cusi, Jianghui Hou, Geoffrey N. Hendy, and Laura Soldati, on behalf of the GENIAL network (Genetics and Environment in Nephrolithiasis Italian Alliance)

Nephrology and Dialysis Unit (G.V., A.T., T.A., D.S.), San Raffaele Hospital Instituto di Ricovero e Cura a Carattere Scientifico, 20132 Milan, Italy; Department of Health Sciences (A.T., A.A., A.M., D.C., L.S.), Università degli Studi of Milan, 20122 Milan, Italy; Institute for Biomedical Technologies (L.M., E.M.), National Research Council, 20090 Segrate (Milan), Italy; Renal Division (J.H.), Washington University, St Louis, Missouri 63110; and Department of Medicine (G.N.H.), Physiology and Human Genetics, McGill University, Montreal, Quebec, H3A1A1 Canada

Background: *CaSR* gene is a candidate for calcium nephrolithiasis. Single-nucleotide polymorphisms (SNPs) encompassing its regulatory region were associated with calcium nephrolithiasis.

Aims: We tested SNPs in the CaSR gene regulatory region associated with calcium nephrolithiasis and their effects in kidney.

Subjects and Methods: One hundred sixty-seven idiopathic calcium stone formers and 214 healthy controls were genotyped for four CaSR gene SNPs identified by bioinformatics analysis as modifying transcription factor binding sites. Strontium excretion after an oral load was tested in 55 stone formers. Transcriptional activity induced by variant alleles at CaSR gene promoters was compared by luciferase reporter gene assay in HEK-293 and HKC-8 cells. CaSR and claudin-14 mRNA levels were measured by real-time PCR in 107 normal kidney medulla samples and compared in patients with different CaSR genotype.

Results: Only rs6776158 (A>G), located in the promoter 1, was associated with nephrolithiasis. Its minor G allele was more frequent in stone formers than controls (37.8% vs 26.4%, $P = .001$). A reduced strontium excretion was observed in GG homozygous stone formers.

Luciferase fluorescent activity was lower in cells transfected with the promoter 1 including G allele at rs6776158 than cells transfected with the A allele.

CaSR mRNA levels were lower in kidney medulla samples from homozygous carriers for the G allele at rs6776158 than carriers for the A allele. Claudin-14 mRNA levels were also lower in GG homozygous subjects.

Conclusions: Minor allele at rs6776158 may predispose to calcium stones by decreasing transcriptional activity of the *CaSR* gene promoter 1 and CaSR expression in kidney tubules. **(*J Clin Endocrinol Metab* 98: 3839–3847, 2013)**

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

Received April 2, 2013. Accepted July 11, 2013.
First Published Online July 17, 2013

* G.V., A.T., and A.A. contributed equally to the study.

Abbreviations: CaSR, calcium-sensing receptor; CI, confidence interval; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GENIAL, Genetics and Environment in Nephrolithiasis Italian Alliance; GFR, glomerular filtration rate; HEK-293, human embryonic kidney cells; HKC-8, human kidney cells; OR, odds ratio; SNP, single-nucleotide polymorphisms; TALH, thick ascending limb of the Henle loop; TRP, tubular reabsorption of phosphate; 5′-UTR, 5′-untranslated regions.

Calcium-sensing receptor (CaSR) is a G protein-coupled receptor that modulates cell activity according to the extracellular calcium concentration. The canonical function of CaSR is to maintain extracellular calcium homeostasis via its role in the regulation of the PTH secretion and renal tubular calcium reabsorption (1–3).

In the kidney, CaSR expression is predominant in the thick ascending limb of the Henle loop (TALH) (1). In this tubular tract and in distal convoluted tubule, it is located in the basolateral membrane of tubular cells and its activation by serum calcium inhibits passive and active calcium reabsorption (4, 5). To inhibit paracellular calcium reabsorption in the TALH, CaSR stimulates the expression of the tight junction protein claudin-14 that blocks the calcium channel made of claudin-16 and claudin-19 in the tight junctions (6, 7). In the collecting duct, CaSR is instead located in the luminal membrane and is therefore sensitive to the increase of calcium in tubular fluid. Here, it likely stimulates urine acidification by intercalated cells and urine dilution by principal cells (8, 9). CaSR is also expressed on the luminal membrane of proximal tubular cells, where its activation by the increase of calcium in glomerular filtrate leads to a more efficient phosphate reabsorption (10). According to these data, in renal tubules the paracrine activities of CaSR give rise to a network of effects that limit the calcium-phosphate precipitation risk induced by the increase of the tubular fluid calcium after CaSR stimulation in TALH. This suggests that an alteration of tubular CaSR activity may amplify the risk of calcium-phosphate precipitation in the tubular lumen and predispose to stone formation.

Consistent with this hypothesis, two polymorphisms (single-nucleotide polymorphisms [SNPs]), rs7652589 and rs1501899, sited in the *CaSR* gene regulatory region, were associated with calcium nephrolithiasis in normocitraturic idiopathic stone formers and in patients with primary hyperparathyroidism (11, 12). These SNPs were also associated with higher serum ionized calcium in patients with primary hyperparathyroidism and serum PTH in idiopathic stone formers (11, 12). The human *CaSR* gene (chr 3q14.3–21) includes seven exons and its regulatory region includes two promoters (promoter 1 and 2) that encode for two alternative 5′-untranslated regions (5′-UTR) having unknown functional differences (13). Therefore, we suggested that calcium stone formers could have an impaired *CaSR* gene promoter activity that decreases CaSR expression in kidney tubules. In keeping with this hypothesis, the present study explored the association of nephrolithiasis with four biallelic SNPs in *CaSR* gene region between rs7652589 and rs1501899, identified by bioinformatics analysis as having a potential effect on *CaSR* gene kidney expression. The transcriptional effect of SNPs significantly associated with stones was then tested using a luciferase reporter assay in human embryonic kidney cells (HEK-293) and human kidney cells (HKC-8). Finally, we evaluated the mRNA expression of CaSR and claudin-14 in kidney medulla from subjects with different CaSR genotypes and tested the regulatory role of CaSR in claudin-based renal calcium handling.

Subjects and Methods

Bioinformatics analysis

To study the CaSR gene region between rs7652589 and rs1501899 (18 240 bp), we selected biallelic SNPs having minor allele frequency >10% from SNP database at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/projects/SNP). SNP activity on transcription factor binding sites was predicted by bioinformatics analysis with the TRANSFAC 12.1 database (14, 15) and prediction tool MATCH11.2, as previously described (11). For each SNP, we determined the possible transcription factor binding sites and defined the position of core regions for the two variant alleles. The score ranges from 0.0 to 1.0, where 1.0 was the exact match; prediction of transcription factor binding sites was considered confident with a core score >0.8. All changes are reported as removed, added, or modified for each SNP. Information about identified transcription factors were obtained from the UniProt database (16).

Patients

One hundred sixty-five idiopathic calcium stone formers and 208 healthy controls were studied. Stone formers and controls were enrolled in the period 1999 to 2003 in the outpatient clinics of the Italian Centres participating to the Genetics and Environment in Nephrolithiasis Italian Alliance (GENIAL) Network, as previously described (11). Calcium stone formers were selected from the GENIAL database because of their normal citrate excretion (>350 mg/24 h) and production of at least two stones in their clinical history. All patients had idiopathic kidney stones that were radio-opaque and/or composed of calcium-oxalate or calcium-phosphate at the chemical or infrared spectrometric analysis. They were not taking drugs affecting electrolyte or citrate handling. Serum concentrations of calcium and potassium had to be normal; urine pH had to be <5.5 in spot morning urine. Controls had negative personal and familial history of kidney stones, normal serum creatinine, and calcium and no evidence of diseases at physical examination.

In both stone formers and controls, we measured serum concentrations of creatinine, calcium, phosphate, sodium, potassium, and PTH and 24-hour urine excretions of creatinine, calcium, phosphate, sodium, potassium, and citrate. Fractional excretion of sodium was calculated with the following formula: [(urine sodium/urine creatinine) × (serum creatinine/serum sodium)], with all values expressed as mmol/L. Tubular reabsorption of phosphate (TRP) was calculated as: [1 − fractional excretion of phosphate]. Calcium excretion was expressed as mmol/24 hours and μmol/100 mL of glomerular filtration rate (GFR) calculated as follows: [(urine calcium/urine creatinine) × serum creatinine]. Vitamin D (25-hydroxycolecalciferol) was

measured by immunochemoluminescence method (DiaSorin) in 54 controls and 63 stone formers of the Milan Unit of the GENIAL network. Intact PTH was measured by an immunochemoluminescence or immunoradiometric method using second-generation kits and was expressed as the percentage of the upper limit of the kit normal range because a centralized laboratory was not feasible.

Fifty-five consecutive stone formers, followed at the San Raffaele Hospital, underwent strontium load test as previously described (11): 2.65 mg/kg of stable strontium was orally administered to patients in water solution (1 g/L) as chloride salt after overnight fast. Blood sample and urine were collected 4 hours after strontium ingestion. Area under the concentration-time curve and fraction of ingested strontium excreted in the urine were calculated.

All patients and control had been previously genotyped for rs7652589 and rs1501899 SNPs (11). All variables were measured at the patient enrollment time period.

The ethical committees of all institutions participating in the GENIAL Network approved the study protocol. All participants provided written informed consent to participate in this study.

Genotyping

DNA was extracted from blood (EDTA as anticoagulant) stored at −80°C with salting out standard protocol, quantified with a spectrophotometer, diluted at final concentration of 10 ng/mL, and stored at −20°C. Genotyping was performed by a specific Taqman SNP genotyping assay on the Applied Biosystems 7500 Fast Real-Time PCR Systems (Applied Biosystems), as previously described (11, 12).

Cells culture

HEK-293 (Cell Bank, IST, Genoa, Italy) and HKC-8 were grown in DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Sigma-Aldrich) at 37°C, 5% CO_2, and 95% humidity.

Site-directed mutagenesis

Human CaSR gene promoter 1 luciferase reporter plasmid was cloned in pGL3-basic plasmid (Promega) (13). Two constructs were designed including promoter 1 with either the wild-type or the minor (G) allele at the rs6776158 SNP (A>G). The construct including the minor allele was generated using the QuickChange XL site-directed kit (Stratagene), according to the manufacturer's instructions. Complementary primers were designed with the mutated base in the middle: forward mutagenic primer 5′GAGGTAGATGT-TATCGCCAT-TTTGCAGAGAAG 3′; reverse mutagenic primer 5′CTTCTCTGCAAAA-TGGCGATA-ACA TCTACCTC 3′ (mutated nucleotides are underlined). Both plasmids were used to transform *Escherichia coli* MAX efficiency DH-5α competent cells (Invitrogen) and plasmid DNA was extracted with QIAGEN Plasmid mini kit (Qiagen). Sequences were confirmed with BigDye terminator v3.1 Cycle Sequencing Kit by ABI 3140XL genetic analyzer (Applied Biosystems, Life Technologies).

Transient transfection of HEK-293 and HKC-8 cells

HEK-293 and HKC-8 cells (2×10^4) per well were grown in 96-well dishes in culture medium and incubated overnight. After 24 hours, cells were transfected with Fugene HD transfection reagent (Promega). Both HEK-293 and HKC-8 were cotransfected (ratio 1:40) with pGL4.74[hRluc/TK] Renilla vector and promoter 1 including wild-type allele or minor allele at its SNPs or empty pGL3basic. The transfection was performed with 0.8 μL Fugene HD transfection reagent and 200 ng total plasmidic DNA (ratio Fugene:DNA = 4:1) in OptiMEM serum-free medium (Invitrogen) up to final volume of 10.8 μL/well. The mixture was incubated for 15 minutes at room temperature and then added to each well.

Luciferase assay

Luciferase assay was performed with Dual-Glo Luciferase Assay System (Promega). Forty-eight hours after the transfection, cells were moved in opaque luminometer-specific 96-well plates with 75 μL DMEM. In each well, 75 μL Dual-Glo Luciferase Reagent was added. After 10 minutes at 20–25°C, firefly luminescence was measured by Glomax Multi Detection System luminometer (Promega) with 5 seconds of integration time. Furthermore, 75 μL Dual Glo Stop and Glo Reagent was added to each well for 10 minutes at 20–25°C and Renilla luminescence was measured by Glomax Multi Detection System luminometer (Promega) with the same integration time. A luminescence value of pGL3-basic vector was subtracted of the pGL4.74 Renilla luminescence value and normalized to negative control (empty pGL3-basic). Data were shown as Luciferase/Renilla ratio. Each experiment was performed in triplicate and repeated in three different days.

Genotyping and quantitative real-time PCR of human renal medulla samples

Kidney medulla samples (10–40 mg) were collected from the healthy portion of kidneys removed from 107 patients hospitalized at the San Raffaele Hospital because of primitive one-sided kidney cancer. None of them had undergone chemotherapy or had metastases or other significant disorders except for the kidney cancer. Immediately after nephrectomy, kidney medulla samples were stored at −80°C. Genomic DNA was extracted from medulla samples by Eurogold Tissue-DNA Mini Kit (Euroclone). Patients were genotyped as described in the previous paragraph.

Total RNA was extracted from kidney medulla samples by PureLink RNA Mini kit (Invitrogen). CaSR and claudin-14 mRNA were estimated by quantitative real-time PCR using specific primers for the CaSR gene (forward: 5′-ATGCCAA-GAAGGGAGAAAGACTCTT-3′; reverse: 5′-TCAGGACACT CCACACACTCAAAG-3′), the claudin-14 gene (forward: 5′-GAAGTTTGAGATTGGCCAGGC-3′; reverse: 5′-GTGCG-GTGTTTGCAGTGGTC-3′) and the housekeeping glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene: (forward: 5′-CTCATGA-CCACAGTCCATGCCATC-3′ and reverse: 5′-CATGCCAGTGAGCTTCCCGTT-3′). Real-time PCR was performed on 100 ng cDNA by SYBR Premix Ex Taq II (Takara Bio Inc) on Applied Biosystems 7500 Fast Real-Time PCR Systems (Applied Biosystems). Amplification condition was 95°C for 5′ and 94°C for 30″, followed by 40 cycles of 94°C for 30″, 57°C for CaSR gene or 60°C for GAPDH and claudin-14 gene for 1′ and, last, 95°C for 15″, 60°C for 1′ and 95°C for 15″. Mean cycle-threshold values of triplicate samples were used for analysis. Data were analyzed by 7500 Fast System SDS Software. CaSR and Claudin-14 mRNA quantities were normalized to the housekeeping gene GAPDH. This study was approved by the San

Table 1. Four SNPs Were Selected in the CaSR Gene Regulatory Region Between rs7652589 and rs1501899

SNP	Contig Position	Alleles	Gene Domain	Transcription Factor	Wild-Type Allele Core Score	Minor Allele Core Score	Transcription Factor Binding Site Modification	Ref.
rs7648041	28394994	C>T	5′	VDR	0.953		Removed	10
rs7648044	28395006	C>T	5′	WT1	0.981		Removed	15
rs6776158	28396995	A>G	P1	PAX-6		0.832	Added	16
rs1048213	28398053	C>T	P2	DBP		0.965	added	17

Abbreviations: DBP, albumin-D-side binding protein; P1 and P2, CaSR gene promoter 1 and promoter 2; VDR, vitamin D receptor; VDRE, vitamin D response element; WT1, transcription factor for Wilms' tumor. They had the minor allele frequency >10% in the National Center for Biotechnology Information SNP database and modified transcription factor binding sites. All transcription factors were found in the kidney. Bioinformatics analysis was performed with TRANSFACT 12.1 database and MATCH 11.2 bioinformatics tool (12, 13).

Raffaele Hospital Ethical Committee. All participants provided written informed consent to participate in this study.

Statistical analysis

Quantitative variables were reported in the text as mean value ± SD and compared between groups by Student's *t* test or one-way ANOVA with Tukey post-hoc test. Allele distribution at the tested CaSR gene SNPs in patients and controls was compared with χ^2 test. Genotype distribution was compared with multinomial logistic regression including CaSR genotype, sex, quartiles of serum phosphate, and quartiles of sodium, citrate, and calcium excretion as independent variables in the model. Relative risk of stones was estimated as the odds ratio (OR) and 95% confidence interval (CI) (95% CI). Statistical analysis was two-tailed and was conducted at $\alpha = .05$ level. Bonferroni correction for multiple comparisons was applied to genotype and allele distribution analysis that was conducted at $\alpha = .0125$ level (17). The statistical power for this α level and a difference of 12% in allele distribution analysis was 86%. Statistical analyses were performed using the SPSS statistical package (IBM).

The linkage disequilibrium between *CaSR* gene SNPs was estimated by calculation of D′ using Haploview 4.2 software (http://www.broad.mit.edu/mpg/haploview). D′ = 1 indicated complete linkage disequilibrium between SNPs. D′ > 0.8 indicated linkage disequilibrium with low probability of recombination between SNPs.

Results

Prediction of SNPs involved in CaSR gene transcription regulation

The bioinformatics analysis of *CaSR* gene region between rs7652589 and rs1501899 identified four SNPs (Table 1): rs7648041 (C>T) was predicted to remove the vitamin D response element (14); rs7648044 (C>T) was found to remove the binding site for Wilms' tumor, a transcription factor specifically expressed in kidney (18); rs6776158 (A>G), located within promoter 1, resulted in adding PAX-6 transcription factor binding site in kidney collecting duct cells (19); rs1048213 (C>T), located in promoter 2, was predicted to add the binding site for albumin-D-side binding protein (20).

Case-control study of the regulatory region SNPs

Stone formers had higher excretion of sodium, calcium, and phosphate compared with controls, whereas serum PTH, serum phosphate, and citrate excretion were lower (Table 2). Allele distribution at the four SNPs identified by bioinformatics analysis respected Hardy-Weinberg equilibrium. Linkage disequilibrium was observed between these SNPs (D′ = 1) and between each of them and rs7652589 or rs1501899 (D′ = 0.79–0.94).

Among these SNPs, only rs6776158 was significantly associated with nephrolithiasis. Its G allele was more frequent in stone formers than in controls (Table 3). Accordingly, GG and AG genotypes were significantly more frequent in stone formers (Table 3). Multinomial logistic regression analysis found that the relative risk of nephro-

Table 2. Variables Measured in Patients and Controls

	Controls	Stone Formers
N, M/F	208 (92/116)	165 (94/71)
Age, y	41 ± 8.9	41 ± 9.9
Body weight, kg	71.1 ± 13.52	69.8 ± 13.64
Serum calcium, mmol/L	2.38 ± 0.134	2.36 ± 0.098
Serum phosphate, mmol/L	1.11 ± 0.184	1.06 ± 0.202[a]
Serum creatinine, mg/dL	0.88 ± 0.163	0.92 ± 0.199
Serum PTH, % of kit upper limit	65.6 ± 19.19	60.1 ± 23.33[a]
Serum vitamin D, ng/mL	28.1 ± 11.8	30.2 ± 9.5
Urine sodium, mmol/24 h	162 ± 54.8	180 ± 63.3[a]
Sodium fractional excretion, %	0.84 ± 0.35	0.8 ± 0.27
Urine calcium, mmol/24 h	5.19 ± 2.67	7.49 ± 3.62[b]
Calcium excretion, μmol/100 mL GFR	3.9 ± 2.57	4.5 ± 1.81[a]
Urine phosphate, mmol/24 h	25.3 ± 8.4	28.3 ± 10.05[a]
TRP, %	83.7 ± 6.51	82.7 ± 6.6
Urine citrate, mg/24 h	597 ± 229.3	667 ± 249.9[c]

Serum vitamin D was measured in a subset of 54 controls and 63 stone formers.

[a] $P < .048$.

[b] $P = .0001$.

[c] $P = .01$.

Table 3. Allele and Genotype Frequency at the *CaSR* Gene SNPs Sited in the Gene Regulatory Region in Normocitraturic Stone Formers and Healthy Controls

SNP	Allele	Genotype	Controls n (%)	Stone Formers n (%)	Odds Ratio (95% CI)	*P*
rs7652589	G		305 (73.3)	204 (61.8)	1	
	A		111 (26.7)	126 (38.2)	1.69 (1.2–2.3)	.0012
rs7648041	C		291 (69.9)	232 (70.3)	1	
	T		125 (30.1)	98 (29.7)	1.02 (0.7–1.4)	.906
rs7648044	C		361 (86.8)	269 (81.5)	1	
	T		55 (13.2)	61 (18.5)	1.49 (1–2.2)	.049
rs6776158	A		306 (73.6)	205 (62.1)	1	
	G		110 (26.4)	125 (37.9)	1.7 (1.2–2.3)	.0008
rs1048213	T		310 (76)	262 (79.4)	1	
	C		98 (24)	68 (20.6)	0.82 (0.6–1.2)	.269
rs1501899	G		312 (75)	204 (61.8)	1	
	A		104 (25)	126 (38.2)	1.87 (1.4–2.6)	.0001
rs7652589		GG	112 (53.8)	65 (39.4)	1	
		GA	80 (38.5)	74 (44.8)	1.6 (1–2.5)	.037
		AA	16 (7.7)	26 (15.8)	2.8 (1.3–5.8)	.004
rs7648041		CC	105 (50.5)	82 (49.7)	1	
		CA	80 (38.5)	68 (41.2)	1.08 (0.7–1.7)	.73
		AA	23 (11)	15 (9.1)	0.9 (0.4–1.8)	.69
rs7648044		CC	153 (73.6)	110 (66.7)	1	
		CT	55 (26.4)	49 (29.7)	1.2 (0.8–1.9)	.36
		TT	0	6 (3.6)	[a]	
rs6776158		AA	113 (54.3)	66 (40)	1	
		AG	80 (38.5)	73 (44.2)	1.6 (1–2.4)	.046
		GG	15 (7.2)	26 (16.8)	3 (1.5–6)	.002
rs1048213		TT	126 (60.6)	103 (62.4)	1	
		CT	69 (33.2)	56 (33.9)	0.96 (0.6–1.5)	.88
		CC	15 (7.2)	6 (3.6)	0.47 (0.2–1.3)	.13
rs1501899		GG	119 (57.2)	67 (40.6)	1	
		GA	74 (35.6)	71 (43.3)	1.7 (1.1–2.7)	.02
		AA	15 (7.2)	27 (16.4)	3.4 (1.7–6.9)	.001

The allele and genotype frequency at rs6776158 SNPs was different in stone formers and controls.

[a] Considering CT and TT together: OR 1.4, 95%CI 0.9–2.2, $P = .148$.

lithiasis was increased in subjects carrying the GG genotype (OR = 5.8, 95% CI 1.7–19.6, $P = .004$) and slightly increased in AG subjects (OR = 1.7, 95% CI 10.9–3.3, $P = .13$). Also, the third (OR = 3.8, 95% CI 1.6–9, $P = .003$) and the highest (the fourth) quartile (OR = 12.7, 95% CI 4.2–38.3, $P = .0001$) of calcium excretion were associated with stones in the regression model.

Phenotypes were related with genotypes at rs6776158 (Table 4). Keeping patients and controls together, subjects carrying the GG genotype at rs6776158 had lower serum phosphate than those carrying the AA genotype and lower TRP than subjects carrying the AG or AA genotype. In stone formers, serum phosphate concentrations and TRP were slightly lower in GG patients.

Strontium excretion after an oral load

Strontium oral load test was carried out in 55 stone formers: 8 patients (5 male/3 female; n = 3 hypercalciuric patients [37.5%]) carrying GG genotype at rs6776158 had a lower proportion of absorbed strontium excreted in urine than 47 patients (24 male/23 female; n = 31 hypercalciuric patients [67.4%]) with AA or AG genotype (1.75 ± 1.19% vs 2.92 ± 2.01%, respectively, $P = .038$). This difference was also observed when fractional strontium excretion was adjusted for body weight and 24-hour calcium excretion ($P = .037$). Intestinal strontium absorption was instead not different in patients with GG genotype vs patients with AA or AG genotype (7.03 ± 2.66 vs 7.55 ± 2.81 mmol L^{-1} min, $P = .62$).

SNP effects on CaSR promoter 1 activity measured with luciferase assay

HEK-293 cells and HKC-8 cells were transiently transfected with plasmids containing *CaSR* gene promoter 1 upstream of the luciferase gene. Two constructs were designed containing A or G allele at the rs6776158 SNP in the promoter 1. Promoter 1 including the G allele showed a lower transcriptional activity than that with the A allele in both HEK-293 (n = 42, 4.86 ± 2.36 vs n = 46, 6.58 ± 3.74, $P = .011$, Student's *t* test) and HKC-8 cells (n = 26, 5.68 ± 2.22 vs n = 26, 7.13 ± 2.84, $P = .045$, Student's *t* test).

Table 4. Phenotypic Characteristics of the Studied Subjects (stone formers and controls together and stone formers alone) Divided According to Their Genotype at the rs6776158 SNP

	All Participants			Stone Formers		
Genotype	**AA**	**AG**	**GG**	**AA**	**AG**	**GG**
N	179	153	41	66	73	26
Age, y	41 ± 9.7	41 ± 9.0	41 ± 9.3	42 ± 10.2	41 ± 9.8	40 ± 9.6
Body weight, kg	71 ± 13.6	70.±13.1	72 ± 15.2	70 ± 13.5	70 ± 12.8	73 ± 16.3
Serum calcium, mmol/L	2.37 ± 0.12	2.37 ± 0.11	2.37 ± 0.07	2.36 ± 0.11	2.37 ± 0.10	2.36 ± 0.07
Serum phosphate, mmol/L	1.11 ± 0.19	1.06 ± 0.19	1.01 ± 0.23[a]	1.09 ± 0.20	1.05 ± 0.19	1.0 ± 0.25
Serum PTH, % of kit upper limit	63.3 ± 22.98	61.1 ± 22.0	62.4 ± 25.45	62.4 ± 27.15	58.8 ± 21.41	60.2 ± 25.9
Serum vitamin D, ng/mL	29.5 ± 9.38 (n = 54)	29.9 ± 10.75 (n = 48)	28.4 ± 11.75 (n = 15)	30.1 ± 7.89 (n = 25)	30.5 ± 10.37 (n = 29)	30.1 ± 11.65 (n = 9)
Urine calcium, mmol/24 h	6.47 ± 3.39	6.82 ± 3.20	6.92 ± 4.11	7.55 ± 3.66	7.52 ± 3.37	7.28 ± 4.29
Calcium excretion, μmol/100 mL GFR	4.29 ± 2.58	4.35 ± 1.75	4.1 ± 1.47	4.4 ± 2.02	4.6 ± 1.76	4.3 ± 1.48
Urine citrate, mg/24 h	623 ± 238.4	634 ± 253.6	634 ± 207.4	661 ± 252	669 ± 263.9	676 ± 211.8
Urine phosphate, mmol/24 h	27.41 ± 10.03	26.15 ± 9.01	30.1 ± 9.4	29.17 ± 11.55	27.13 ± 8.97	29.1 ± 8.69
TRP, %	83.3 ± 7.17	83.8 ± 4.55	79.6 ± 8.8[b]	82.9 ± 7.46	83.4 ± 4.42	80.3 ± 8.97
Urine sodium, mmol/24 h	179 ± 63	175 ± 53.9	188 ± 73	179 ± 68.7	177 ± 53.3	192 ± 76.5
Sodium fractional excretion, %	0.8 ± 0.33	0.82 ± 0.29	0.83 ± 2.2	0.78 ± 0.3	0.79 ± 0.26	0.85 ± 0.23
Stone rate, stone number/ patients/y				0.8 ± 0.95	0.8 ± 1.3	1.5 ± 2.1

One-way ANOVA with Tukey test to comparison between groups. GFR is the glomerular filtration rate (mL/min). Serum vitamin D was measured in a subset of 54 controls and 63 stone formers.

[a] $P = .031$ vs AA homozygotes.

[b] $P = .005$ vs AG heterozygotes and $P = .015$ vs AA homozygotes.

CaSR mRNA expression in the kidney

CaSR expression as mRNA was measured by real-time PCR in 107 healthy kidney medulla samples from patients nephrectomized because of primitive cancer. Twelve patients carried the GG genotype, 49 the AG genotype, and 48 the AA genotype at rs6776158. CaSR mRNA levels, reported as a ratio with GAPDH mRNA, were significantly lower in GG homozygotes (n = 12, 1.7 ± 1.17) than subjects carrying AG or AA genotypes taken as a unique group (n = 95, 2.75 ± 2.18, $P = .0158$; Figure 1). Kidney medulla content of claudin-14 mRNA was also lower in GG homozygotes than in subjects carrying AG or AA genotypes (0.75 ± 0.58 vs 1.36 ± 1.83, $P = .0198$). CaSR and claudin-14 expression levels were positively correlated across all genotypes of rs6776158 ($r = 0.293$, $P = .003$; Figure 2).

Discussion

In the present study calcium nephrolithiasis was associated with the rs6776158 SNP, located within *CaSR* gene promoter 1. The in vitro model using in transfected HEK-293 and HKC-8 cells (13, 21) showed that the minor G allele at rs67780158 caused a decrease in the transcriptional efficiency of the promoter 1. Accordingly, the level of the CaSR mRNA was lower in kidney medulla samples isolated from homozygotes for the G allele than those carrying AA or AG genotypes.

These findings suggest that SNPs decreasing tubular CaSR expression may be involved in calcium nephrolithiasis. The signaling mechanism of CaSR has recently been demonstrated in studies showing that its agonists stimulate CaSR translocation from the endoplasmic reticulum to the plasma membrane (22). This agonist-driven insertional signaling could amplify the negative effect of the expression-decreasing SNPs on the CaSR activity.

Decreased CaSR expression may improve the tubular capability to reabsorb calcium (2) as revealed by the lower strontium excretion after an acute load. Strontium is often used as a surrogate marker to study intestinal and renal calcium handling (1, 23). Furthermore, the medullary expression of CaSR was correlated with that of claudin-14, whose expression was also decreased in homozygotes for the G allele at rs6776158. This confirms previous observations that CaSR deactivation in TALH may favor paracellular calcium reabsorption by decreasing claudin-14 expression (6, 7). Through these mechanisms, CaSR down-regulation could increase calcium concentration in renal interstitium predisposing to hydroxyapatite precipitation in the Randall's plaque (24). Calcium-oxalate stones then develop after precipitation of urinary calcium oxalate on Randall's plaques sited at the papilla surface (25).

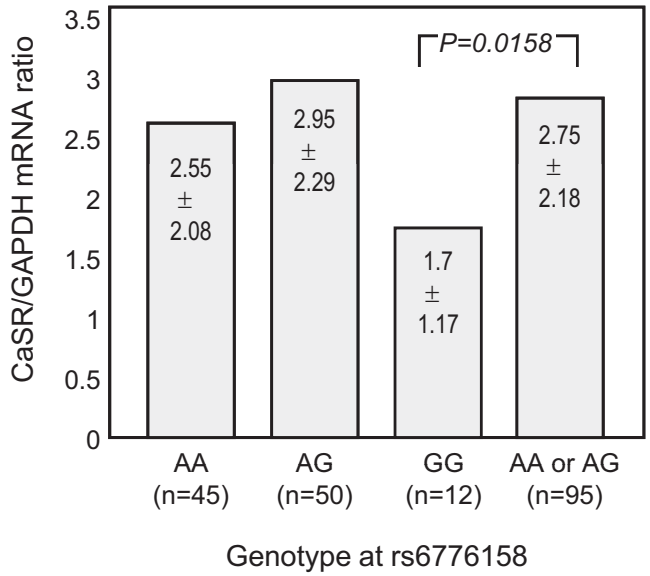

Figure 1. CaSR mRNA content in kidney medulla associated with the genotype at CaSR gene rs6776158 SNP (A>G). Kidney medulla content of CaSR mRNA was normalized to the GAPDH housekeeping gene (CaSR/mRNA GAPDH mRNA ratio). GG homozygotes had a lower level of CaSR mRNA compared with subjects with AA and AG genotype. The difference was significant when GG homozygotes (n = 12) were compared with subjects with AA or AG genotype (n = 97) taken as a whole (1.7 ± 1.17 vs 2.75 ± 2.18, $P = .016$).

Deficient expression may also impair CaSR capability to stimulate urine acidification and dilution in collecting ducts and phosphate retention in proximal tubules (8–10). These defects expose patients to the risk of calcium-phosphate precipitation in the tubular fluid that may lead to macroprecipitate retention in Bellini ducts and calcium-phosphate stone production (25). The association of rs6776158 SNP with lower phosphate serum concentrations and TRP is consistent with this view and confirms the role of CaSR in renal phosphate handling (10, 26). However, we cannot explain this role in detail because we did not measure fibroblast-growth-factor 23 that is crucial to regulate tubular phosphate reabsorption (27).

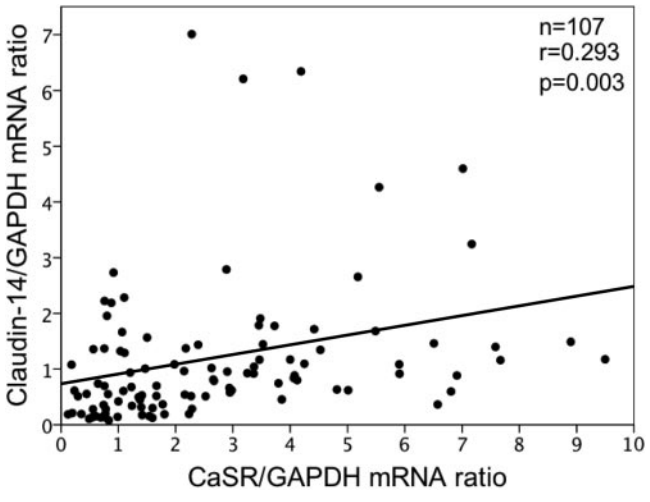

Figure 2. CaSR expression as mRNA was positively correlated with claudin-14 mRNA in 107 healthy medulla kidney samples from patients who had been nephrectomized for kidney cancer. CaSR and claudin-14 mRNAs were normalized to GAPDH mRNA.

CaSR down-regulation has been hypothesized to be involved in arterial wall calcification. The use of calcimimetic drugs prevents the artery calcification in laboratory animals and the differentiation of cultured vascular muscle cells to osteoblast-like cells (28, 29). In this process, calcimimetic drugs stimulate vascular smooth muscle cells to produce antiosteogenic factors such as matrix-gla-protein that inhibits calcium-phosphate precipitation in soft tissues and urine (29). These observations suggest that CaSR protects against interstitial hydroxyapatite precipitation in the Randall's plaque with a mechanism that could be similar to that occurring for soft tissue calcification. Nephrolithiasis could therefore be considered as the renal consequence of a systemic predisposition to calcify soft tissues. A few studies concerning this hypothesis have been performed and observed increased frequency of mitral valve annulus calcification, hypertension, and atherosclerosis in stone formers (30–32).

In addition to SNPs decreasing CaSR expression, the gain-of-function R990G SNP was associated with calcium nephrolithiasis in patients with primary hyperparathyroidism, but not in those with idiopathic stones (33, 34). It was also associated with primary hypercalciuria (35, 36). Interestingly, for unknown reasons neither R990G nor rs6776158 was correlated with total serum calcium and PTH in normocalcemic subjects (11, 33, 36). Therefore, the CaSR's role in stone formation and calcium homeostasis appears complex and bivalent, because its up-regulation and down-regulation could both promote stone formation in patients with different phenotypic backgrounds. rs6776158 could predispose normocitraturic patients to calcium stones, whereas R990G SNP could act in patients with primary hyperparathyroidism by enhancing their tendency to high urine calcium concentrations. Another bivalent effect of CaSR is exemplified by the antiosteoporotic activity of both calcimimetic and calcilytic drugs (37, 38).

According to our findings, CaSR genotype may be included among factors predisposing to stones. Among them, high sodium intake and, consequently, excretion are typical of patients with recurrent stones as in our sample (39). Another predisposing factor is protein intake that may increase net phosphate and acid intake (40). *CaSR* genotype at rs6776158 could amplify the stone-promoting effect of proteins limiting TRP and urine acidification (40).

In conclusion, SNPs decreasing CaSR gene promoter 1 activity may increase susceptibility to calcium nephrolithiasis by decreasing the renal expression levels of CaSR

and claudin-14 calcium in the TALH. Because a low CaSR expression was also observed in calcified arteries, calcium nephrolithiasis may represent the renal alteration of a systemic tendency to soft tissue calcification.

Acknowledgments

We thank Dr Martin Hewison (Cedars-Sinai Medical Center, Los Angeles) for kindly providing the HCK-8 cells.

Address all correspondence and requests for reprints to: Giuseppe Vezzoli, MD, Unità di Nefrologia e Dialisi, Istituto Scientifico Universitario San Raffaele, Via Olgettina 60, 20132 Milano, Italy. E-mail: vezzoli.giuseppe@hsr.it.

This work was supported by grants from Italian Research Ministry (L.S.) and National Institutes of Health (J.H.).

This article has been written with the contribution of the GENIAL network researchers who should be regarded as coauthors of it: Vera Paloschi (San Raffaele Hospital, Milan), Elena Dogliotti, Caterina Brasacchio (Università degli Studi of Milan), Giacomo Dell'Antonio (San Raffaele Hospital, Milan), Francesco Montorsi, Roberto Bertini, Piera Bellinzoni, Giorgio Guazzoni (Vita Salute University, San Raffaele Hospital, Milan), Loris Borghi, Angela Guerra, Franca Allegri, Andrea Ticinesi, Tiziana Meschi, Antonio Nouvenne (University of Parma), Antonio Lupo, Antonia Fabris (University-Hospital of Verona), Giovanni Gambaro (Catholic University, Rome), Pasquale Strazzullo, Domenico Rendina, Giampaolo De Filippo (Federico II University, Naples), Maria Luisa Brandi, Emanuele Croppi, Luisella Cianferotti (University of Florence), Alberto Trinchieri (Manzoni Hospital, Lecco), Renata Caudarella (Villa Maria-Health Science Foundation, Lugo, Ravenna), Adamasco Cupisti (University of Pisa), and Franca Anglani, Dorella Del Prete (University of Padua).

Disclosure Summary: The authors have nothing to disclose.

References

1. **Hofer A, Brown EM.** Extracellular calcium sensing and signaling. *Nature*. 2003;4:530–538.
2. **Riccardi D, Brown EM.** Physiology and pathophysiology of the calcium-sensing receptor in the kidney. *Am J Physiol Renal Physiol*. 2010;298:F485–F499.
3. **Brown EM, Gamba G, Riccardi D, et al.** Cloning and characterization of an extracellular Ca(2+)-sensing receptor from bovine parathyroid. *Nature*. 1993;366:575–580.
4. **Wang W, Li C, Kwon TH, et al.** Reduced expression of renal Na+ transporters in rats with PTH-induced hypercalcemia. *Am J Physiol Renal Physiol*. 2004;286:F534–F545.
5. **Blankenship KA, Williams JJ, Lawrence MS, McLeish KR, Dean WL, Arthur JM.** The calcium-sensing receptor regulates calcium absorption in MDCK cells by inhibition of PMCA. *Am J Physiol*. 2001;280:815–822.
6. **Toka HR, Al-Romaih K, Koshy JM, et al.** Deficiency of the calcium-sensing receptor in the kidney causes parathyroid hormone-independent hypocalciuria. *J Am Soc Nephrol*. 2012;23:1879–1890.
7. **Gong Y, Renigunta V, Himmerkus N, et al.** Claudin-14 regulates renal Ca^{++} transport in response to CaSR signalling via a novel microRNA pathway. *EMBO J*. 2012;31:1999–2012.
8. **Renkema KY, Velic A, Dijkman HB, et al.** The calcium-sensing receptor promotes urinary acidification to prevent nephrolithiasis. *J Am Soc Nephrol*. 2009;20:1705–1713.
9. **Procino G, Mastrofrancesco L, Tamma G, et al.** Calcium-sensing receptor and aquaporin 2 interplay in hypercalciuria-associated renal concentrating defect in humans. An in vivo and in vitro study. *PLoS ONE*. 7:e33145.
10. **Ba J, Brown D, Friedman PA.** Calcium-sensing receptor regulation of PTH-inhibitable proximal tubule phosphate transport. *Am J Physiol Renal Physiol*. 2003;285:F1233–F1243.
11. **Vezzoli G, Terranegra A, Arcidiacono T, et al.** Calcium kidney stones are associated with a haplotype of the calcium-sensing receptor gene regulatory region. *Nephrol Dial Transplant*. 2010;25:2245–2252.
12. **Vezzoli G, Scillitani A, Corbetta S, et al.** Polymorphisms at the regulatory regions of the CASR gene influence stone risk in primary hyperparathyroidism. *Eur J Endocrinol*. 2011;164:421–427.
13. **Canaff L, Hendy GN.** Human calcium-sensing receptor gene. **Vitamin D response elements in promoters P1 and P2 confer transcriptional responsiveness to 1,25-dihydroxyvitamin D.** *J Biol Chem*. 2002;277:30337–30350.
14. **Matys V, Kel-Margoulis OV, Fricke E, et al.** TRANSFAC and its module TRANSCompel: transcriptional gene regulation in eukaryotes. *Nucleic Acids Res*. 2006;34:374–378.
15. **Kel AE, Gössling E, Reuter I, Cheremushkin E, Kel-Margoulis OV, Wingender E.** MATCH: A tool for searching transcription factor binding sites in DNA sequences. *Nucleic Acids Res*. 2003;31:3576–3579.
16. **UniProt Consortium.** The universal protein resource (UniProt). *Nucleic Acids Res*. 2008;36:D190–D195.
17. **Balding DJ.** A tutorial on statistical methods for population association studies. *Nat Rev Genet*. 2006;7:781–791.
18. **Morrison AA, Viney RL, Saleem MA, Ladomery MR.** New insights into the function of the Wilms tumor suppressor gene WT1 in podocytes. *Am J Physiol Renal Physiol*. 2008;295:F12–F17.
19. **Tchapyjnikov D, Li Y, Pisitkun T, Hoffert JD, Yu MJ, Knepper MA.** Proteomic profiling of nuclei from native renal inner medullary collecting duct cells using LC-MS/MS. *Physiol Genom*. 2010;40:167–183.
20. **Khatib ZA, Inaba T, Valentine M, Look AT.** Chromosomal localization and cDNA cloning of the human DBP and TEF genes. *Genomics*. 1994;23:344–351.
21. **Canaff L, Zhou X, Hendy GN.** The proinflammatory cytokine, interleukin-6, up-regulates calcium-sensing receptor gene transcription via Stat1/3 and Sp1/3. *J Biol Chem*. 2008;283:13586–13600.
22. **Grant MP, Stepanchick A, Cavanaugh A, Breitwieser GE.** Agonist-driven maturation and plasma membrane insertion of calcium-sensing receptors dynamically control signal amplitude. *Sci Signal*. 2011;4:ra78.
23. **Vezzoli G, Baragetti I, Zerbi S, et al.** Strontium absorption and excretion in normocalciuric subjects: relation to calcium metabolism. *Clin Chem*. 1998;44:586–590.
24. **Breiderhoff T, Himmerkus N, Stuiver M, et al.** Deletion of claudin-10 (Cldn10) in the thick ascending limb impairs paracellular sodium permeability and leads to hypermagnesemia and nephrocalcinosis. *PNAS*. 2012;109:14241–14246.
25. **Coe FL, Evan A, Worcester E.** Pathophysiology-based treatment of idiopathic calcium kidney stones. *Clin J Am Soc Nephrol*. 2011;6:2083–2092.
26. **Kestenbaum B, Glazer NL, Köttgen A, et al.** Common genetic variants associate with serum phosphorus concentration. *J Am Soc Nephrol*. 2010;21:1223–1232.
27. **Prie D, Urena Torres P, Friedlander G.** Latest findings in phosphate homeostasis. *Kidney Int*. 2009;75:882–889.
28. **Alam M, Kirton JP, Wilkinson FL, et al.** Calcification is associated with loss of functional calcium sensing receptor in vascular smooth muscle cells. *Cardiovasc Res*. 2009;81:2660–2668.
29. **Koleganova N, Piecha G, Ritz E, Schmitt CP, Gross ML.** A calci-

mimetic (R-568), but not calcitriol, prevents vascular remodeling in uremia. *Kidney Int*. 2009;75:60–71.
30. **Celik A, Davutoglu V, Sarica K, et al.** Relationship between renal stone formation, mitral annular calcification and bone resorption markers. *Ann Saudi Med*. 2010;30:301–305.
31. **Domingos F, Serra A.** Nephrolithiasis is associated with an increased prevalence of cardiovascular disease. *Nephrol Dial Transplant*. 2011;26:864–868.
32. **Reiner AP, Kahn A, Eisner BH, et al.** Kidney stones and subclinical atherosclerosis in young adults: the CARDIA study. *J Urol*. 2011; 185:920–925.
33. **Vezzoli G, Terranegra A, Arcidiacono T, et al.** R990G polymorphism of calcium-sensing receptor does produce a gain-of-function and predispose to primary hypercalciuria. *Kidney Int*. 2007;71: 1155–1162.
34. **Corbetta S, Eller-Vainicher C, Filopanti M, et al.** R990G polymorphism of the calcium-sensing receptor and renal calcium excretion in patients with primary hyperparathyroidism. *Eur J Endocrinol*. 2006;155:687–692.
35. **Scillitani A, Guarnieri V, Battista C, et al.** Primary hyperparathyroidism and the presence of kidney stones are associated with different haplotypes of the calcium-sensing receptor. *J Clin Endocrin Metab*. 2007;92:277–283.
36. **Vezzoli G, Tanini A, Ferrucci L, et al.** Influence of calcium-sensing receptor gene on urinary calcium excretion in stone-forming patients. *J Am Soc Nephrol*. 2002;13:2517–2523.
37. **Chattopadhyay N, Quinn SJ, Kifor O, Ye C, Brown EM.** The calcium-sensing receptor (CaR) is involved in strontium ranelate-induced osteoblast proliferation. *Biochem Pharmacol*. 2007;74:438–447.
38. **Arey BJ, Seethala R, Ma Z, et al.** A novel calcium-sensing receptor antagonist transiently stimulates parathyroid hormone secretion in vivo. *Endocrinology*. 2005;146:2015–2022.
39. **Yun SJ, Ha YS, Kim WT, Kim YJ, Lee SC, Kim WJ.** Sodium restriction as initial conservative treatment for urinary stone disease. *J Urol*. 2010;184:1372–1376.
40. **Curhan GC, Willett WC, Rimm EB, Stampfer MJ.** A prospective study of dietary calcium and other nutrients and the risk of symptomatic kidney stones. *N Engl J Med*. 1993;328:833–838.