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Primary Hyperparathyroidism and the Presence of Kidney Stones Are Associated with Different Haplotypes of the Calcium-Sensing Receptor

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Introduction: Three single-nucleotide polymorphisms in the calcium-sensing receptor gene (*CASR*) encoding the missense substitutions A986S, R990G, and Q1011E have been associated with normal variation in extracellular calcium homeostasis, both individually and in haplotype combination. The aim of this study was to examine haplotype associations in primary hyperparathyroidism (PHPT).

Patients and Methods: Patients with sporadic PHPT (n = 237) were recruited from endocrine clinics and healthy controls (n = 433) from a blood donor clinic, and levels of serum calcium, albumin, and PTH were measured. In PHPT patients, urinary calcium/creatinine clearances and bone mineral density at spine and femoral neck were measured and the presence of kidney stones and vertebral fractures identified. The *CASR* single-nucleotide polymorphisms were haplotyped by allele-specific sequencing.

Results: Four haplotypes (ARQ, SRQ, AGQ, and ARE) of eight were observed, in keeping with significant linkage disequilibrium, but haplotype frequencies did not show significant Hardy-Weinberg disequilibrium. The SRQ haplotype was more common in PHPT (125 of 474 alleles) than in controls (170 of 866 alleles, $P = 0.006$) and showed a significant ($P = 0.006$) gene-dosage effect. There was no significant association between haplotype and bone mineral density or fractures, but association with kidney stones was significant ($P = 0.0007$). In the stone-forming subgroup, the SRQ haplotype was underrepresented and AGQ overrepresented. Patients bearing the AGQ haplotype had an odds ratio of 3.8 (95% confidence interval, 1.30–11.3) for presentation with renal stones compared with the rest.

Conclusion: Our data indicate that the *CASR* SRQ haplotype is significantly associated with PHPT in our population. Within the PHPT patient population, the AGQ haplotype is significantly associated with kidney stones. **(*J Clin Endocrinol Metab* 92: 277–283, 2007)**

SERUM CALCIUM CONCENTRATIONS are under significant genetic control in normal individuals (1, 2) and are maintained within a narrow range by PTH. The relationship between serum calcium and PTH levels is mediated by the calcium-sensing receptor (CASR), a G protein-coupled cell-surface glycoprotein expressed in parathyroid gland and renal tubular cells (3). In the kidney, its activation induces increased calcium excretion (3). Inactivating or activating mutations of the CASR cause familial hypocalciuric hypercalcemia (FHH) or autosomal dominant hypocalcemia, respectively (4, 5), emphasizing the central role of the CASR in blood calcium homeostasis. Three single-nucleotide polymorphisms (SNPs) in exon 7 of the CASR gene (*CASR*), all encoding nonconservative amino acid changes (A986S, R990G, and Q1011E) and clustered in the CASR carboxyl-terminal tail, have been described (6). These polymorphisms have been found to be predictive of serum calcium concentrations in normal Caucasian populations either individually (7, 8) or in haplotype combination (9).

Thus, the *CASR* is a candidate gene for association with disorders of calcium regulation (7). Vezzoli *et al.* (10) found that *CASR* R990G was associated with urinary calcium excretion in a population of Caucasian hypercalciuric stone formers. Comparisons of normal individuals and patients with primary hyperparathyroidism (PHPT), a common disorder of calcium homeostasis affecting 0.3% of the general

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Abbreviations: BMD, Bone mineral density; $Ca_{alb\text{-}adj}$, albumin-adjusted serum calcium; CASR, calcium-sensing receptor; CI, confidence interval; DXA, dual-energy x-ray absorptiometry; FHH, familial hypocalciuric hypercalcemia; GLM, general linear modeling; OR, odds ratio; PHPT, primary hyperparathyroidism; SNP, single-nucleotide polymorphism.

population (11) and 2.1% of postmenopausal women (12), that seek association with the *CASR* SNPs have been conducted only on a small scale and without significant positive findings (13, 14).

These observations prompted us to undertake a more definitive study in a larger group of patients with sporadic PHPT in comparison with healthy control subjects, looking at the development of the disease and its sequelae, osteoporosis, fractures, and renal stones.

Subjects and Methods

Subjects

From April 2001 to December 2004, 312 patients with PHPT were recruited at two referral centers, Rome and San Giovanni Rotondo. In all patients, serum calcium, albumin, creatinine, PTH, prolactin, gastrin, and calcitonin and urinary metanephrines, 5-hydroxy-indoleacetic acid, and calcium/creatinine clearance ratio (calcium clearance) were measured. Criteria for exclusion from this study included hormonal and biochemical results or a familial history suggestive of multiple endocrine neoplasia types 1 or 2, FHH, or familial isolated hyperparathyroidism. Patients with parathyroid carcinoma were likewise excluded. A total of 237 (Rome, 47, and San Giovanni Rotondo, 190) sporadic PHPT patients were included: 41 males [aged 57 ± 15 yr (mean ± SD)] and 196 females [aged 58 ± 13 yr (43 premenopausal, aged 40 ± 10 yr, and 153 postmenopausal, aged 63 ± 8 yr)]. One hundred fifty-three subjects underwent surgery for parathyroidectomy, and a histological diagnosis of adenoma or hyperplasia was made in 127 and 26, respectively.

Control subjects were recruited from a blood donor clinic after exclusion of those taking drugs or affected by diseases influencing bone metabolism. In all, serum calcium, albumin, creatinine, and PTH were measured. Subjects whose results were outside the normal reference interval were excluded. The 433 control subjects remaining included 205 males [aged 38 ± 10 yr (mean ± SD)] and 228 females [aged 40 ± 11 yr (167 premenopausal, aged 35 ± 9 yr, and 61 postmenopausal, aged 53 ± 4 yr)].

All subjects gave informed consent for the study that was approved by the ethical committees of Casa Sollievo della Sofferenza Hospital and Roma University Department of Clinical Science.

Genetic analysis

Genomic DNA was extracted from peripheral white blood cells using standard techniques. A 282-bp fragment of *CASR* exon 7 was amplified as before (9), and the three *CASR* SNPs were determined by direct sequencing. For the doubly heterozygous subjects, phase was resolved using allele-specific amplification at the first locus and heterozygosity detection at the second, and the four observed haplotypes (ARQ, SRQ, AGQ, and ARE) were assigned as described before (9).

Chemistries

Serum calcium, albumin, and creatinine and urinary calcium and creatinine were measured by standard colorimetric techniques. Serum intact PTH was measured by Allegro immunoradiometric assay (Nichols Institute Diagnostics, San Juan Capistrano, CA) with intra- and interassay coefficients of variation of 5.1 and 8.2%, respectively, and a reference interval of 10–72 pg/ml.

Bone mineral density (BMD), fractures, and renal stones

BMD was measured by dual-energy x-ray absorptiometry (DXA) (Hologic, Waltham, MA) at the spine (DXA L2-L4, *in vivo* precision 1.0%) and femoral neck (*in vivo* precision 2.3%). Individual BMD values were expressed as SD units (Z-values) in relation to age-matched reference populations of each center (15). Conventional spinal radiographs in lateral (T4–L4) and anteroposterior projection (L1–L4) were obtained with a standardized technique. Vertebral fractures were diagnosed by visual inspection using the semiquantitative method described by Genant *et al.* (16). History of nonvertebral fractures was collected from patient medical records.

Subjects with a presumptive diagnosis of PHPT were routinely assessed for a history of renal stones, including renal ultrasound examination. Those who had renal colic also underwent urography and plain-film radiography. Documentation of urographic and plain-film radiographic results and surgical removal of stones was obtained by review of medical records. Subjects were considered as stone formers if 1) they had a positive history for renal stones according to ultrasound examination, urography, plain-film radiography, or surgical removal, as recorded in the patient chart, or 2) renal stones were diagnosed by these procedures in either asymptomatic and symptomatic patients at physical examination.

Statistical analysis

Linkage and Hardy-Weinberg disequilibria were assessed using the Genetic Data Analysis program [Lewis, P. O., and D. Zaykin, computer program for the analysis of allelic data (version 1.0d16c)] based on standard methods described by Weir (17).

The χ^2 test was used for evaluating the association of tri-locus haplotypes with disease and, within the PHPT group, with renal stone or fracture phenotype. Differences among biochemical parameters both in patients and in controls in relation to the tri-locus haplotypes were performed by one-way ANOVA and Duncan *post hoc* comparisons or Student's *t* test for unpaired data, as appropriate. Excluded from the associational analyses were the single instances of homozygosity at R990G (AGQ/AGQ) and Q1011E (ARE/ARE) in the control group and in the patient group, respectively, leaving 432 and 236 subjects in each cohort. General linear modeling (GLM) was applied, both in the patient group and in the control group, to evaluate the contribution of haplotypes to serum calcium levels after correction for the following variables: age, sex, serum creatinine, and serum PTH.

In the PHPT group, bivariate association between haplotypes (expressed as ordinal levels 0, 1, and 2, according to the number of *CASR* haplotype alleles) and biochemical data were performed by Kendall's τ-b correlation. In the PHPT group, GLM was performed to evaluate the potential association between haplotypes and BMD measured at spine and femoral neck after correction for the following variables: body mass index (BMI, kg/m^2), serum PTH, and calcium clearance.

Logistic regression analysis was performed to evaluate the influence of the different haplotypes in predicting disease status in all subjects. Moreover, in the PHPT group, it was used to evaluate the influence of the different haplotypes in predicting the presence of renal stones after correction for the following variables: age, sex, serum PTH, and calcium clearance.

Unadjusted odds ratios (OR) for the presence of the 986S genotype in PHPT cohorts and controls were calculated for this study and two other published studies (13, 14) and then pooled using a random-effects model as described (18). A 95% confidence interval (CI) was calculated for the summary OR using the same method. Heterogeneity across the studies was assessed by visual inspection, and a formal statistical test was done as described (19). The hypothesis that the studies were not heterogeneous was rejected at $P < 0.05$.

Data are expressed as mean ± SE, unless otherwise indicated. A *P* value of less than 0.05 was considered significant. All nongenetic analyses were performed using the SPSS version 12.0 statistical package (SPSS Inc., Chicago, IL).

Results

As expected, linkage disequilibrium was observed for all three pairwise comparisons between the three clustered missense polymorphisms. However, haplotype distributions within genotypes showed no significant departure from Hardy-Weinberg equilibrium in PHPT patients or controls.

The tri-locus haplotype frequencies for the *CASR* were significantly different between PHPT and control groups ($\chi^2 = 8.39$; $P = 0.038$), with most of the difference attributable to the SRQ haplotype (Table 1). Analysis of SRQ haplotype alone revealed a significantly higher frequency in PHPT patients than in controls ($\chi^2 = 7.72$; $P = 0.006$) and a significant trend for SRQ with disease status ($\chi^2 = 7.58$; $P = 0.006$).

TABLE 1. Individual haplotype frequencies in PHPT and control groups

	CASR haplotype frequency (%)			
	ARQ	SRQ	AGQ	ARE
PHPT[a]	316/474 (66.7)	125/474 (26.4)[b]	19/474 (4.0)	14/474 (3.0)
Control	623/866 (71.9)	170/866 (19.6)	43/866 (5.0)	30/866 (3.5)

[a] Significantly different: $P = 0.039$ for $\chi^2 = 8.39$ by contingency table analysis with three degrees of freedom.
[b] Significantly greater than in controls: $P = 0.006$ for $\chi^2 = 7.72$ with one degree of freedom.

After adjustment for the other covariates, serum calcium (albumin-adjusted, $Ca_{alb\text{-}adj}$) was significantly and positively associated with SRQ ($P = 0.0001$) in controls and negatively with AGQ ($P = 0.026$) (Table 2). In the control group, there was a significant trend for $Ca_{alb\text{-}adj}$ with SRQ (F for trend = 21.6; $P = 0.0001$) but not in the patient group (Table 2). Age, serum creatinine, and PTH were not different in either controls or patients stratified by haplotype (Table 2).

Bivariate correlation between haplotypes and key biochemical parameters in the PHPT group (Table 3) showed that SRQ was significantly and negatively associated with calcium clearance ($r = -0.199$; $P = 0.0001$), whereas AGQ was significantly and positively associated ($r = 0.140$; $P = 0.013$). No significant correlations were seen with serum creatinine or PTH.

When stratified by haplotype, no significant differences were seen for BMD, whether measured at lumbar spine or femoral neck, or for fractures (Table 4).

In an analysis of the PHPT cohort alone (Table 5), the kidney stone phenotype revealed significant association with haplotype ($\chi^2 = 17.2$; $P = 0.0007$). The SRQ haplotype was significantly and negatively associated with stone-former status, and a significant trend for SRQ *vs.* non-stone phenotype ($\chi^2 = 6.25$; $P = 0.012$) was observed, whereas the AGQ haplotype was significantly and positively associated with the stone phenotype (Table 5).

Comparison of PHPT patients with and without kidney stones is summarized in Table 6. Patients with stones were somewhat younger, more likely to be male, and had higher serum PTH and urinary calcium indices, but serum calcium was higher in stone-formers. Logistic regression analysis showed that the *CASR* AGQ haplotype was positively associated with renal stones ($P = 0.015$) after correction for age, sex, serum PTH, and calcium clearance. Overall, PHPT subjects with AGQ had a 3.8-fold higher risk of developing renal stones (95% CI, 1.30–11.3) (Fig. 1). In the regression model, covariates significantly associated with stone phenotype were age ($P = 0.028$) and calcium clearance ($P = 0.031$). Moreover, logistic regression analysis showed that the *CASR* AGQ haplotype was positively associated with renal stones ($P = 0.010$) after correction for age, sex, serum PTH, and serum calcium.

TABLE 2. Clinical characteristics according to tri-locus haplotype

Allele	Haplotype	Clinical data					
		No. of subjects	Sex (male/female)	Age (yr)	$Ca_{alb\text{-}adj}$ (mg/dl)	PTH (pg/ml)	Creatinine (mg/dl)
PHPT patients							
ARQ	X/X	27	2/25	61 ± 2	11.12 ± 0.17	133 ± 16	0.95 ± 0.05
	X/ARQ	102	19/83	57 ± 1	11.14 ± 0.08	170 ± 15	0.91 ± 0.02
	ARQ/ARQ	107	20/87	58 ± 1	11.15 ± 0.08	187 ± 18	0.96 ± 0.03
SRQ	X/X	133	26/107	58 ± 1	11.20 ± 0.07	192 ± 17	0.97 ± 0.03
	X/SRQ	81	13/68	57 ± 2	11.04 ± 0.10	152 ± 12	0.88 ± 0.02
	SRQ/SRQ	22	2/20	60 ± 3	11.17 ± 0.18	140 ± 20	0.93 ± 0.05
AGQ	X/X	217	37/180	58 ± 1	11.12 ± 0.06	168 ± 11	0.93 ± 0.02
	X/AGQ	19	4/15	58 ± 3	11.35 ± 0.19	234 ± 59	1.05 ± 0.08
ARE	X/X	224	39/185	58 ± 1	11.14 ± 0.06	176 ± 11	0.94 ± 0.02
	X/ARE	12	2/10	59 ± 3	11.27 ± 0.25	134 ± 20	0.96 ± 0.07
Controls							
ARQ	X/X[c]	29	11/18	39 ± 2	9.37 ± 0.05[a]	37 ± 3	0.82 ± 0.03
	X/ARQ	183	82/101	40 ± 1	9.23 ± 0.02	41 ± 1	0.83 ± 0.01
	ARQ/ARQ	220	111/109	38 ± 1	9.17 ± 0.02	40 ± 1	0.83 ± 0.01
SRQ	X/X	282	138/144	39 ± 1	9.17 ± 0.02	40 ± 1	0.83 ± 0.01
	X/SRQ	130	58/72	40 ± 1	9.26 ± 0.03[b]	41 ± 1	0.82 ± 0.01
	SRQ/SRQ	20	8/12	37 ± 3	9.46 ± 0.07[c,d]	38 ± 4	0.84 ± 0.04
AGQ	X/X	391	188/203	39 ± 1	9.22 ± 0.02[e]	40 ± 1	0.82 ± 0.01
	X/AGQ	41	16/25	42 ± 2	9.11 ± 0.05	39 ± 2	0.86 ± 0.02
ARE	X/X	402	190/212	39 ± 1	9.21 ± 0.02	40 ± 1	0.83 ± 0.01
	X/ARE	30	14/16	41 ± 2	9.25 ± 0.05	44 ± 3	0.81 ± 0.03

Data are shown as mean ± SE. Data were analyzed by one-way ANOVA and Duncan *post hoc* comparison or *t* test for unpaired data, as appropriate. $Ca_{alb\text{-}adj}$ was evaluated by GLM after correction for age, sex, serum creatinine, and PTH and Sidak *post hoc* comparison. X indicates any other haplotype.
[a] $P = 0.001$ for X/X *vs.* X/ARQ and ARQ/ARQ.
[b] $P = 0.007$ for X/SRQ *vs.* X/X.
[c] $P = 0.0001$ for SRQ/SRQ *vs.* X/X.
[d] $P = 0.015$ for SRQ/SRQ *vs.* X/SRQ.
[e] $P = 0.026$ for X/X vs. X/AGQ.

TABLE 3. Bivariate correlation analysis in the PHPT cohort

Clinical data	Allele			
	ARQ	SRQ	AGQ	ARE
$Ca_{alb\text{-}adj}$ (mg/dl)	0.053	−0.102	0.063	0.041
Creatinine (mg/dl)	0.028	−0.065	0.091	−0.021
PTH (pg/ml)	0.051	−0.080	0.079	−0.025
Ca_u/Cr_u (mg/mg)	0.037	−0.082	0.052	0.050
Calcium excretion	0.075	−0.138[a]	0.088	0.052
Calcium clearance	0.120[b]	−0.199[c]	0.140[d]	0.022

Data were assessed by Kendall's τ-b correlation. Calcium excretion was calculated as [urinary calcium (Ca_u)/urinary creatinine (Cr_u)] × serum creatinine (mmol/liter glomerular filtrate).

[a] $P = 0.010$.
[b] $P = 0.028$.
[c] $P = 0.0001$.
[d] $P = 0.013$.

Discussion

PHPT is characterized by dysregulated PTH secretion and parathyroid cell growth (20). The pivotal role of the parathyroid CASR in orchestrating calcium homeostasis and regulating parathyroid secretory function and cell proliferation is emphasized by the clinical phenotypes of humans with *CASR*-inactivating mutations (4). Heterozygous loss-of-function mutations give rise to FHH in which the lifelong hypercalcemia is typically asymptomatic (21). Although overt hyperparathyroidism is not normally a part of this benign phenotype, members of some FHH kindreds (or in some cases those classified as having familial isolated hyperparathyroidism) present atypically with hyperparathyroidism, and surgical removal of the adenoma or hyperplastic glands can be curative (22–26). The homozygous condition manifests as neonatal severe hyperparathyroidism characterized by marked parathyroid hypercellularity (21).

Mouse models of FHH and neonatal severe hyperparathyroidism have been generated by heterozygous or homozygous deletions, respectively, of the *Casr* gene (27). The homozygous null mutants die shortly after birth with severe hypercalcemia and hyperparathyroidism. However, genetic ablation of PTH is sufficient to rescue the lethal $Casr^{-/-}$ phenotype (28). Adult $Pth^{-/-}$, $Casr^{-/-}$ mice exhibit an expanded range of serum and urine calcium values, highlighting the importance of the CASR in fine control of blood calcium levels and renal calcium excretion, even in the absence of PTH (28).

The $Pth^{-/-}Casr^{-/-}$ double knockouts have markedly enlarged parathyroid glands, confirming the importance of normal serum calcium concentrations and CASR in the inhibition of parathyroid cell proliferation (20). Whether abnormal parathyroid proliferation in PHPT is always the result of alteration in calcium-regulated PTH secretion has been the subject of some debate (29). In a transgenic mouse model of primary hyperparathyroidism in which the cyclin D1 protooncogene is targeted to parathyroid cells (30), abnormal parathyroid proliferation precedes dysregulation of the calcium-PTH axis (31). In this particular model, then, it could be concluded that the proliferative defect need not occur as a consequence of the defective coupling of secretory control of PTH to serum calcium. Although overexpression of cyclin D1 occurs in 20–40% of PHPT tumors, no differences were found between PHPT and control groups in the allele frequency of an *Nci*I polymorphism in the cyclin D1 gene (32). It is likely that the mechanisms underlying the development and progression of primary parathyroid tumors are heterogeneous, and no single mechanism predominates (33).

In healthy adult Caucasian populations, the association of the A986S polymorphism in the CASR carboxyl-terminal tail with normal variation in extracellular calcium concentration, both individually (7, 8) and in haplotype combination with the neighboring R990G and Q1011E polymorphisms (9), suggests that *CASR* is a good candidate for association studies in PHPT populations. Nevertheless, *CASR* A986S, the most common polymorphic variant in Caucasians, was not found to be significantly associated with PHPT in two earlier studies, albeit with smaller numbers of subjects (13, 14). Nevertheless, a trend toward a higher frequency of the 986S variant (AS+SS *vs.* AA-wild-type) in PHPT is evident in those data sets [in Miedlich *et al.* (13), 20 of 50 patients (40%) *vs.* 29 of 102 controls (29%); in Cetani *et al.* (14), 41 of 103 (40%) patients *vs.* 45 of 148 controls (30%)]. Those frequencies are comparable to our study [103 of 237 patients (43.5%) *vs.* 150 of 433 controls (34.6%)]. It is not surprising that metaanalysis of the pooled data are clearly consistent with association between A986S and PHPT status ($P = 0.002$), the pooled OR being 1.49 (95% CI, 1.15–1.93) (see Fig. 2).

TABLE 4. BMD (Z-DXA at spine and femoral neck) and fractures according to haplotype

Allele	Haplotype	Clinical data			
		n	Total Fx (y/n)	Z-(L2–L4)	Z-FN
ARQ	X/X	24	8/16	−0.075 ± 0.334	−0.258 ± 0.243
	X/ARQ	91	38/53	−0.634 ± 0.174	−0.542 ± 0.130
	ARQ/ARQ	102	36/66	−0.550 ± 0.157	−0.485 ± 0.117
SRQ	X/X	125	46/79	−0.518 ± 0.145	−0.459 ± 0.109
	X/SRQ	71	28/43	−0.643 ± 0.199	−0.556 ± 0.146
	SRQ/SRQ	21	8/13	−0.254 ± 0.352	−0.371 ± 0.256
AGQ	X/X	200	74/126	−0.547 ± 0.114	−0.498 ± 0.084
	X/AGQ	17	8/9	−0.298 ± 0.425	−0.213 ± 0.337
ARE	X/X	208	80/128	−0.552 ± 0.112	−0.493 ± 0.082
	X/ARE	9	2/7	0.050 ± 0.561	−0.127 ± 0.439

Data are presented as mean ± SE. The total number (n) of PHPT patients was 217 (91.6% of patients enrolled) after exclusion of those on medications or affected by diseases that influence bone metabolism. X indicates any other haplotype. Z-values were analyzed by GLM after correction for BMI, PTH, and calcium/creatinine clearance ratio. FN, Femoral neck; Fx, fractures; y/n, yes/no.

TABLE 5. Frequency of a positive kidney stone phenotype according to haplotype

Allele	Haplotype	Clinical data						
		n	Sex (male/female)	Age (yr)	Kidney stones (y/n)	PTH (pg/ml)	Calcium excretion[d] (mmol/liter GF)	Calcium clearance
ARQ	X/X	24	1/23	60 ± 3	10/14	138 ± 18	0.065 ± 0.008	0.023 ± 0.002
	X/ARQ	96	19/77	57 ± 1	54/42	174 ± 16	0.070 ± 0.05	0.024 ± 0.001
	ARQ/ARQ	105	20/85	58 ± 1	57/48	188 ± 19	0.073 ± 0.003	0.026 ± 0.001
SRQ	X/X	130	26/104	58 ± 1	78/52[a]	194 ± 18	0.078 ± 0.005	0.027 ± 0.001[b]
	X/SRQ	74	13/61	57 ± 2	36/38	156 ± 14	0.063 ± 0.005	0.021 ± 0.001
	SRQ/SRQ	21	1/20	60 ± 3	7/14	142 ± 20	0.065 ± 0.008	0.023 ± 0.003
AGQ	X/X	207	36/171	58 ± 1	105/102	171 ± 11	0.070 ± 0.003	0.024 ± 0.001
	X/AGQ	18	4/14	57 ± 3	16/2[e]	242 ± 62	0.093 ± 0.015[c]	0.032 ± 0.004[d]
ARE	X/X	215	38/177	58 ± 1	113/102	179 ± 12	0.070 ± 0.003	0.025 ± 0.001
	X/ARE	10	2/8	59 ± 5	8/2	138 ± 24	0.080 ± 0.013	0.026 ± 0.004

Data are shown as mean ± SE. Total number (n) of PHPT patients was 225 (94.9% of patients enrolled) after exclusion of those on medications or affected by diseases that influence kidney stone formation. X indicates any other haplotype. Calcium excretion was calculated as (urinary calcium/urinary creatinine) × serum creatinine. GF, Glomerular filtrate; y/n, yes/no.

[a] $\chi^2 = 6.34$; $P = 0.042$ for X/X *vs.* SRQ/SRQ.

[b] $P = 0.003$ for X/X *vs.* X/SRQ, by one-way ANOVA and Duncan *post hoc* comparison.

[c] $P = 0.017$ for X/AGQ *vs.* X/X, by t test for unpaired data.

[d] $P = 0.005$ for X/AGQ *vs.* X/X, by t test for unpaired data.

[e] By Fisher exact test = 9.70; $P = 0.002$ for X/AGQ *vs.* X/X.

In our previous study of a large cohort of Caucasian subjects (9), the tri-locus SNP cluster (A986S, R990G, and Q1011E) was a significant predictor of blood ionized calcium levels. Subjects with SRQ and ARE haplotypes were relatively hypercalcemic, whereas those with AGQ were hypocalcemic, relative to subjects with the wild-type ARQ haplotype. In the present study, although the association of serum calcium with haplotype we have observed before is also evident in our controls, it is not seen in the patient group. We suggest that the tri-locus haplotypes, if they are functionally important in the normal homeostasis of serum calcium, become less so once inhibition of parathyroid cell proliferation and differentiation is lost in PHPT.

In the present study, we found that the SRQ haplotype is significantly more common in PHPT patients than in controls and shows a significant gene dosage effect with disease status. Furthermore, in PHPT stone formers, the lower frequency of SRQ and the higher frequency of AGQ, together with the significant association of SRQ (negatively) and AGQ (positively) with calcium clearance, supports a counterbalancing function of these polymorphic variants, the 986S being relatively inactivating and the 990G activating relative to wild-type ARQ. The association of higher serum calcium in healthy subjects bearing the SRQ haplotype and lower serum calcium in healthy subjects bearing the AGQ haplotype argues for a functional role for these polymorphic variants. This hypothesis is in keeping with the observation by Vezzoli *et al.* (10) of a higher frequency of 990G variant in hypercalciuric stone formers than normocalciuric ones or controls. The same investigators recently presented preliminary data demonstrating that polymorphic variant 990G of the *CASR* gene results in CASR gain-of-function, which should result in greater inhibition of calcium reabsorption in distal tubular cells of the kidney and cause hypercalciuria (34).

In our PHPT patients, logistic regression analysis showed that subjects bearing the AGQ haplotype have a 3.8-fold higher risk of developing renal stones after correcting for covariates. In PHPT subjects, we calculated calcium clearances as a measure of calcium excretion, taking into account

TABLE 6. Clinical characteristics of PHPT patients with and without stones

Clinical data	Kidney stone	
	Yes	No
Sex (male/female)	25/96	15/89
Age (yr)	55 ± 1.24	61 ± 1.19[a]
$Ca_{alb\text{-}adj}$ (mg/dl)	11.37 ± 0.10[b]	10.96 ± 0.08
PTH (pg/ml)	183 ± 14.9	169 ± 17.4
Creatinine (mg/dl)	0.98 ± 0.03[c]	0.88 ± 0.02
GFR (ml/min)	72.2 ± 1.7	70.7 ± 1.8
Ca_u/Cr_u (mg/mg)	0.33 ± 0.02	0.29 ± 0.02
Calcium excretion (mmol/liter GF)	0.078 ± 0.005[d]	0.063 ± 0.003
Calcium clearance	0.027 ± 0.001[c]	0.022 ± 0.001

Data are shown as mean ± SE. Differences were analyzed by t test for unpaired data. Glomerular filtration rate (GFR) was calculated according to the Cockroft-Gault equation by GLM analysis after adjusting for age. Calcium excretion was calculated as [urinary calcium (Ca_u)/urinary creatinine (Cr_u)] × serum creatinine. GF, Glomerular filtrate.

[a] $P = 0.001$.

[b] $P = 0.002$.

[c] $P = 0.003$.

[d] $P = 0.005$.

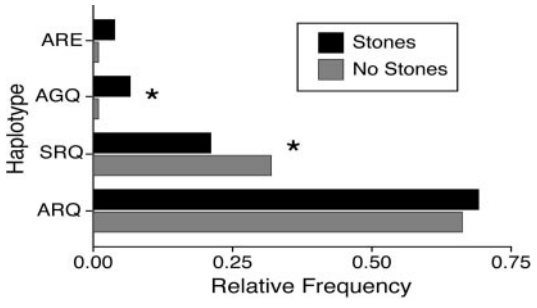

FIG. 1. Relative haplotype frequencies in the PHPT cohort stratified by the presence (*black bar*) or absence (*gray bar*) of renal stones. The *error bar* represents the 95% CI for the proportion. The difference in the distribution of frequencies of all four haplotypes was significant ($\chi^2 = 17.2$; $P = 0.0007$); *, $P < 0.05$, presence *vs.* absence of renal stones for SRQ and AGQ groups.

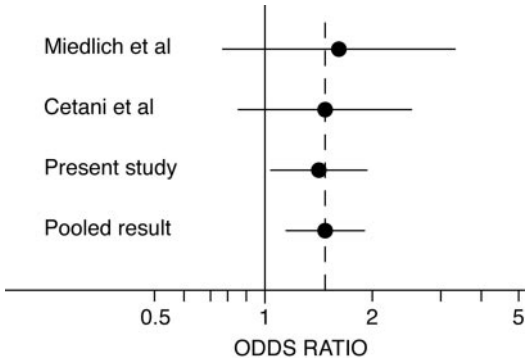

FIG. 2. Odds ratios for the 986S genotype in PHPT cohorts *vs.* controls in three different studies. Estimates and 95% CI for each study together with the pooled estimates are shown. The *solid vertical line* shows an odds ratio of 1. The *dashed vertical line* shows the pooled estimate of 1.49, with a Cochrane Q statistic for heterogeneity of 0.14 (not significant).

both renal function and serum calcium levels. Correlation between AGQ haplotype and clearance suggest that predisposition to development of renal stones could be a result of increased calcium clearance, but whether it is exclusively mediated by a decreased inhibitory effect of the AGQ-containing CASR on suppression of renal calcium reabsorption with subsequent hypercalciuria cannot be assessed from our data, especially given the ascertainment bias introduced by selection of cohorts from PHPT populations presenting at hospital clinics. However, the significant association of AGQ with stones after adjustment for calcium clearance suggests the possibility of AGQ-mediated actions that are independent of the effect on hypercalciuria.

Although the CASR is involved in bone metabolism either directly through osteoclast activity (35, 36) or indirectly through its effect on regulation of PTH secretion and calcium metabolism (3), the reports of association between measures of bone quality (DXA and heel ultrasound) and the *CASR* haplotype variants have been inconsistent (37–40). In a study of 230 Hungarian postmenopausal women, *CASR* 986S was not significantly associated with BMD (41) or with vertebral fractures in 219 Italian postmenopausal women (42). Likewise, in the present study, we did not observe any association between *CASR* haplotype and bone mass by DXA or with fractures.

In conclusion, the data show a significant association of SRQ haplotype in PHPT patients, thus suggesting it as a susceptibility marker for the development of PHPT. Moreover, this is the first evidence that PHPT patients bearing the AGQ haplotype are at greater risk, whereas those having the SRQ haplotype are at lesser risk, of developing renal stones.

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