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Blood Ionized Calcium Is Associated with Clustered Polymorphisms in the Carboxyl-Terminal Tail of the Calcium-Sensing Receptor

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Blood ionized calcium (iCa) is a quantitative trait subject to genetic influence. iCa is maintained in a narrow range through the action of the calcium-sensing receptor (CASR) controlling PTH secretion and calcium excretion. A CASR single nucleotide polymorphism (SNP) prevalent in Caucasian populations (A986S) has shown significant association with iCa in a cohort of young women, but association with the neighboring SNPs, R990G and Q1011E, has not been examined. We studied 377 unrelated adults (184 men and 193 women) recruited as healthy adults from a blood donor clinic. The subjects were not taking any medications, nor did they have disorders of calcium metabolism. Relative frequencies for the CASR 986S, 990G, and 1011E minor alleles were 24%, 4%, and 3% respectively. At the A986S locus, subjects with the AA genotype had significantly lower iCa (P = 0.0001) than subjects with one or two S alleles (mean ± SE, 1.221 ± 0.003 *vs.* 1.239 ± 0.003 mmol/liter). For the R990G site, subjects with the RR genotype had higher iCa than those with one copy of the 990G allele (1.230 ± 0.002 *vs.* 1.213 ± 0.007 mmol/liter; P = 0.032). With respect to the 1011 locus, iCa was lower in QQ genotype subjects than in the QE group (1.227 ± 0.002 *vs.* 1.255 ± 0.008 mmol/liter; P = 0.002). After resolution of phase for the doubly heterozygous subjects, analysis was conducted on haplotypes across all three loci. As expected, subjects with SRQ and ARE haplotypes are relatively hypercalcemic, and those with AGQ are hypocalcemic, relative to subjects with the common ARQ haplotype. Multiple regression analysis with clinical covariates (age, sex and menopausal status, creatinine, and PTH) showed that 16.5% of the total variance in iCa may be explained, and the seven CASR haplotypes contribute significantly (P < 0.0001) and substantially (49.1% of the explained variance) to the model, with the following corrected iCa means: ARQ/AGQ, 1.21 ± 0.01; ARQ/ARQ, 1.22 ± 0.01; ARQ/SRQ, 1.24 ± 0.01; SRQ/AGQ, 1.24 ± 0.03; SRQ/SRQ, 1.25 ± 0.01; ARQ/ARE, 1.25 ± 0.01; and SRQ/ARE, 1.27 ± 0.01. Our data confirm the association between iCa and the A986S locus and suggest that R990G and Q1011E are also predictive. Given the significant between-population variations in frequency of variant alleles in this CASR SNP cluster, tri-locus haplotyping may prove to be more informative in studies of association between variation in CASR and disease. (*J Clin Endocrinol Metab* 89: 5634–5638, 2004)

SERUM CALCIUM IS a quantitative trait subject to genetic control. Studies in mono- and dizygotic twins have demonstrated that the variance in total calcium due to genetic effects is between 50% and 78% (1, 2). Serum calcium levels are maintained in a narrow range under the control of PTH. The relationship between ionized calcium (iCa) and PTH is mediated by the calcium-sensing receptor (CASR), a G protein-coupled plasma membrane glycoprotein that is expressed in the parathyroids and in renal tubular cells, where it regulates calcium excretion (3). Single inactivating or activating mutations of the CASR gene cause hypercalcemic or hypocalcemic disorders, known as familial hypocalciuric hypercalcemia and autosomal dominant hypocalcemia, respectively (4, 5). This suggests a pivotal role of CASR gene variants in extracellular calcium regulation. Heath *et al.* (6) described three clustered polymorphisms in the intracellular tail encoded by exon 7, inducing nonconservative amino acid changes. Among these, the $Ala^{986}Ser$ (A986S) polymorphism has been associated with population differences in serum calcium, suggesting that it is a significant determinant of serum calcium levels, at least in North American cohorts (7, 8). Some have confirmed this effect in smaller Caucasian samples (9, 10), but others have not (11–14). The nearby nonconservative polymorphisms, $Arg^{990}Gly$ (R990G) and $Gln^{1011}Glu$ (Q1011E), are more common in Asian (5, 15) and Afro-American (5) populations, respectively, but have not been carefully studied in relation to ionized calcium (16). The aim of this study was to evaluate the frequency of the A986S, R990G, and Q1011E polymorphisms and their association with ionized serum calcium in a large, well characterized, adult cohort.

Abbreviations: CASR, Calcium-sensing receptor; iCa, ionized calcium; SNP, single nucleotide polymorphism.

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Subjects and Methods

Subjects

Between April 2001 and September 2003, Caucasian adults were prospectively recruited from a blood donor clinic in San Giovanni Rotondo (Apulia, Italy). Subjects were excluded from additional study if they were taking medication or known to be affected by diseases affecting calcium metabolism. In those deemed eligible, iCa, serum creatinine, and PTH were measured; measurements were excluded if results were outside the normal range. The final cohort of 377 subjects consisted of 184 men (median age, 43 yr; range, 18–65 yr), 137 premenopausal women (median age, 37 yr; range, 18–52 yr), and 56 postmenopausal women (median age, 53 yr; range, 48–65 yr). All subjects came from southeastern Italy, and most were residents of the Apulia provinces. All patients gave informed consent, and the study, designed according to Helsinki Declaration II, was approved by the ethical committee of Casa Sollievo della Sofferenza Hospital.

Genotyping

Genomic DNA was extracted from peripheral white blood cells using standard proteinase K-sodium dodecyl sulfate digestion and phenol-chloroform extraction or the Wizard Genomic DNA Purification Kit (Promega Corp., Madison WI). A fragment of exon 7 of 282 bp encompassing the three single nucleotide polymorphisms (SNPs) was amplified by PCR using the following primers: forward, 5′-CAGATGCAAG-CAGAAGGTCA-3′; and reverse, 5′-ACAAGTGCTGGGGACAACTC-3′. PCR conditions were initial denaturation at 95 C for 5 min; 35 cycles at 95 C for 30 sec, 60 C for 30 sec, and 72 C for 30 sec; and a final extension at 72 C for 10 min. Approximately 100 ng genomic DNA were used for each sample. PCR controls with no DNA as template were used to monitor potential reaction contamination. After purification with a Gel Band Purification Kit (Amersham Biosciences, Piscataway, NJ), direct sequencing of the 282-bp product was performed with an ABI BigDye Terminator Cycle Sequencing Ready Reaction kit and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Warrington, UK).

Phasing of double heterozygotes

For subjects with ASRGQQ and ASRRQE genotypes, phasing was undertaken with allele-specific amplification. For both genotypes, genomic DNA was subjected to PCR with an allele-specific forward primer 5′-GCTTTGATGAGCCTCAGAAGAACG-3′ and reverse primer 5′-CTGTTTCCTGGACGGTCAGATCT-3′, so that only the copy with the A986-containing haplotype was amplified. PCR conditions were: initial denaturation at 95 C for 12 min; then 35 cycles of denaturation at 95 C for 30 sec, annealing at 52 C for 30 sec, and extension at 72 C for 30 sec; followed by final extension at 72 C for 7 min. The product was mixed with equal amounts of DNA similarly amplified from a subject homozygous across all three loci (AARRQQ), and the mixture was subjected to denaturing HPLC at 65 C. For subjects with the downstream variant allele in *cis* (ARQ/SGQ or ARQ/SRE), a shift in profile consistent with heterozygosity would be expected. For subjects with the downstream variant allele in *trans* (SRQ/AGQ or SRQ/ARE), the profile of the admixture remained unchanged.

Chemistries

Serum iCa (corrected to pH 7.4) was measured by ion-selective electrode with an intraassay coefficient of variation of 3.4% and a reference interval of 1.12–1.32 mmol/liter (AVL LIST GmbH Medizintechnik, Graz, Austria). Serum creatinine was measured by a standard colorimetric technique, and serum intact PTH was determined by the Allegro immunoradiometric assay (Nichols Institute Diagnostics, San Juan Capistrano, CA), which has intra- and interassay coefficients of variation of 5.1% and 8.2%, respectively, and a reference interval of 10–72 pg/ml.

Statistical analysis

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

Differences among the measured analytes in relation to the single locus genotypes and tri-locus haplotypes were performed by one-way ANOVA and Duncan *post hoc* comparisons or *t* test for two-sample unpaired data. Excluded from this associational analysis was the single instance of 990G homozygosity (AGQ/AGQ), leaving 376 subjects in the cohort. Differences in iCa and other parameters stratified by genetic group were evaluated by one-way parametric ANOVA after assessment for unequal sample variance.

To evaluate the effects of different covariates on calcium, general linear modeling was used, based on iCa, corrected to pH 7.4, as the single dependent variable, genotype as an independent fixed predictor, and age, creatinine, PTH, and gender/menopausal status as covariates. Data are expressed as the mean ± SE unless otherwise indicated. $P < 0.05$ was considered significant. All nongenetic analyses were performed using the SPSS version 11.5 statistical package (SPSS, Inc., Chicago, IL).

Results

Clinical characteristics

For the entire cohort, there was a narrow distribution of serum iCa values, as expected. The overall mean (±SD) for all 376 subjects was 1.228 ± 0.041 mmol/liter, with a range of 1.12–1.32 mmol/liter and no significant deviation from a parametric distribution (by Kolmogorov-Smirnov test, P = 0.180). Mean serum creatinine was 0.88 ± 0.17 (±SD) mg/dl (range, 0.47–1.35 mg/dl), and immunoreactive PTH was 40.3 ± 13.3 (±SD) pg/ml (range, 11.5–72 pg/ml). In a comparison of male and pre- and postmenopausal female groups, we found no differences in iCa (1.225 ± 0.003 *vs.* 1.226 ± 0.004 *vs.* 1.241 ± 0.006 mmol/liter, respectively; P = 0.07) once values were corrected for creatinine, PTH, and age, and stratification according to gender and postmenopausal status was not further considered.

Single locus genotypes

Genotype frequencies for the three CASR loci are shown in Table 1. There was no evidence of Hardy-Weinberg disequilibrium, but the 986 and 990 loci showed small, but significant, pairwise linkage disequilibrium. The coefficient (0.0104 ± 0.0018) was significantly different ($\chi^2 = 12.9$; $P <$ 0.007) from zero. Exact test analysis of the tri-locus disequilibrium using permutation methods gave probabilities close to 0.05, suggesting a trend toward nonrandom association of alleles.

Examining the cohort stratified according to single genotype (Table 1), we found no genotype-specific differences with respect to age, serum PTH, or creatinine. However, subjects with the common AA genotype at the A986S locus had significantly lower iCa than subjects with one or two S alleles (1.221 ± 0.003 *vs.* 1.239 ± 0.003 mmol/liter; P = 0.0001), and ANOVA showed a significant trend for iCa, with S as the hypercalcemic allele (F for trend = 10.48; $P = 0.0013$). For the R990G locus, however, subjects with the common RR genotype had higher iCa than those with a 990G allele (1.230 ± 0.002 *vs.* 1.213 ± 0.007 mmol/liter; $P = 0.032$). When grouped by Q1011E genotype, subjects with the common QQ genotype had lower iCa than those with the QE genotype (1.227 ± 0.002 *vs.* 1.255 ± 0.008 mmol/liter; P = 0.002; Table 1).

Tri-locus haplotypes

Haplotyping of the double heterozygotes revealed that all of the minor alleles were present in the *trans* configuration.

Of the four ASRGQQ subjects, all were SRQ/AGQ, and all five ASRRQE subjects were haplotyped as SRQ/ARE. Analysis of all haplotypes by exact test for Hardy-Weinberg disequilibrium did not reach significance ($P = 0.37$), but the small numbers of mutant homozygotes seriously limit the power of any test for nonrandom association of alleles.

Examining the sample stratified according to tri-locus haplotypes (Table 2), we found no haplotype-specific differences with respect to age, serum PTH, or creatinine. Subjects homozygous for the ARQ/ARQ genotype had significantly lower iCa than subjects with none ($P = 0.0001$) or one ($P = 0.038$) of the common alleles, and ANOVA showed a significant trend for iCa, with ARQ as the hypocalcemic haplotype (F for trend = 17.27; $P = 0.0001$). Stratified by SRQ haplotype, subjects without any SRQ allele had significantly lower iCa than subjects with one ($P = 0.001$) or two ($P = 0.004$) alleles, and ANOVA showed a significant trend for iCa, with SRQ as the hypercalcemic haplotype (F for trend = 10.48; $P = 0.001$). For the AGQ haplotype, however, subjects with the AGQ haplotype had lower iCa than those without it ($P = 0.032$). When grouped by ARE haplotype, subjects with the ARE haplotype had higher iCa than those without it ($P = 0.002$).

Haplotypes and multiple regression analysis

Mean iCa values according to the seven tri-locus haplotype groups are given in Table 3. After adjustment for the other covariates, haplotypes with ARE or SRQ and without AGQ alleles were associated with higher adjusted iCa concentrations ($P < 0.05$) compared with ARQ/ARQ or AGQ/ARQ genotypes (Fig. 1). Multiple regression analysis showed that all variables together accounted for 16.5% of the total variance in iCa (F = 7.24; $P = 0.0001$), and haplotype was a significant predictor (F = 5.94; $P = 0.0001$), accounting for nearly half (49.1%) of the explained variance for iCa in this model.

Discussion

Twin studies indicate that the variance of serum calcium is primarily due to genetic effects (1, 2). The CASR protein has a central role in mediating the relationship among iCa, PTH, and renal handling of calcium. Indeed, activating and inactivating mutations of CASR induce opposite effects on serum and urinary calcium, resulting in hypocalciuric hypercalcemia and hypercalciuric hypocalcemia, respectively (4, 5). These relatively rare disorders suggest that SNPs might be

TABLE 1. Clinical characteristics according to single-locus genotype[a]

Locus[b]	Genotype	Clinical characteristic				
		n[c]	Age (yr)	iCa (mmol/liter)	PTH (pg/ml)	Creatinine (mg/dl)
A986S	AA	223	43.0 ± 0.8	1.221 ± 0.003[d,e]	39.7 ± 0.9	0.87 ± 0.01
	AS	126	43.3 ± 1.1	1.237 ± 0.004	41.6 ± 1.2	0.89 ± 0.02
	SS	27	40.4 ± 2.6	1.247 ± 0.007	39.5 ± 2.8	0.91 ± 0.03
R990G	RR	345	42.8 ± 0.7	1.230 ± 0.002[f]	40.3 ± 0.7	0.88 ± 0.01
	RG	31	43.8 ± 2.1	1.213 ± 0.007	40.0 ± 2.6	0.90 ± 0.03
Q1011E	QQ	355	43.0 ± 0.6	1.227 ± 0.002[g]	40.3 ± 0.7	0.88 ± 0.01
	QE	21	41.4 ± 2.6	1.255 ± 0.008	41.4 ± 2.9	0.90 ± 0.03

[a] Mean ± SE.
[b] No evidence was found for Hardy-Weinberg disequilibrium at any of the three loci, analyzed by Fisher exact test (17).
[c] Number of subjects.
[d] $P = 0.001$ for AA *vs.* AS; [e] $P = 0.004$ for AA *vs.* SS, by one-way ANOVA and Duncan *post hoc* comparison.
[f] $P = 0.032$ for RR *vs.* RG, by t test for unpaired data.
[g] $P = 0.002$ for QQ *vs.* QE, by t test for unpaired data.

TABLE 2. Clinical characteristics according to tri-locus haplotype[a]

Allele	Haplotype	Clinical data				
		n[b]	Age (yr)	iCa (mmol/liter)	PTH (pg/ml)	Creatinine (mg/dl)
ARQ	X/X[c]	36	40.4 ± 2.3	1.251 ± 0.006[d,e]	40.2 ± 2.4	0.92 ± 0.03
	X/ARQ	160	43.5 ± 0.9	1.232 ± 0.003	41.2 ± 1.1	0.89 ± 0.01
	ARQ/ARQ	180	42.9 ± 0.9	1.220 ± 0.003	39.6 ± 1.0	0.87 ± 0.01
SRQ	X/X	223	43.0 ± 0.8	1.221 ± 0.003[f,g]	39.7 ± 0.9	0.87 ± 0.01
	X/SRQ	126	43.3 ± 1.1	1.237 ± 0.004	41.6 ± 1.2	0.89 ± 0.02
	SRQ/SRQ	27	40.4 ± 2.6	1.247 ± 0.007	39.5 ± 2.8	0.91 ± 0.03
AGQ	X/X	345	42.8 ± 0.7	1.230 ± 0.002[h]	40.3 ± 0.7	0.88 ± 0.01
	X/AGQ	31	43.8 ± 2.1	1.213 ± 0.007	40.0 ± 2.6	0.90 ± 0.03
ARE	X/X	355	43.0 ± 0.6	1.227 ± 0.002[i]	40.3 ± 0.7	0.88 ± 0.01
	X/ARE	21	41.4 ± 2.6	1.255 ± 0.008	41.4 ± 2.9	0.90 ± 0.03

[a] Mean ± SE.
[b] Number of subjects.
[c] X = Any other haplotype.
[d] $P = 0.038$ for X/X *vs.* X/ARQ; [e] $P = 0.0001$ for X/X *vs.* ARQ/ARQ, by one-way ANOVA and Duncan *post hoc* comparison.
[f] $P = 0.001$ for X/X *vs.* X/SRQ; [g] $P = 0.004$ for X/X *vs.* SRQ/SRQ, by one-way ANOVA and Duncan *post hoc* comparison.
[h] $P = 0.032$ for X/X *vs.* X/AGQ, by t test for unpaired data.
[i] $P = 0.002$ for X/X *vs.* X/ARE, by t test for unpaired data.

predictive of extracellular iCa in healthy populations (18, 19). In a cohort of premenopausal Caucasian females, serum calcium was found to be associated with the A986S polymorphism (7, 8). This SNP may thus be a genetic determinant of serum calcium and a candidate for predisposition to diseases of mineral metabolism. The two other polymorphisms clustered in the intracellular tail of CASR, namely R990G and Q1011E, are less common in Caucasians and more frequent in Asiatics and Africans, respectively (5, 16), but significant association with serum calcium levels has not been studied in detail.

In our larger cohort of healthy male and female adults, we found that the 986S allele is relatively common (28.8%), and the similar findings of Vezzoli *et al.* (10) suggest that it may exist at a higher background frequency in some Italian populations than in other groups (7–9, 11, 12), including the Toronto cohort (16.3%; $P = 0.006$). The association of increased iCa with 986S, previously reported by others and us (7–10), is confirmed in the present study. Three other groups have examined the relationship between extracellular calcium and the A986S polymorphism without finding a significant association. In a German cohort, investigators analyzed corrected serum calcium in a smaller number of subjects (n = 102) and found no correlation. However, they reported a trend toward higher calcium levels in subjects with one or two 986S alleles (12). More recently, Young *et al.* (13) and Bollerslev *et al.* (14) analyzed calcium data for 102 and 252 postmenopausal women, respectively, and reported no difference in circulating calcium levels based on A986S genotype. In the latter study, the older age of the cohort may also be a factor, but it is interesting to note that the relative difference between groups with and without a 986S allele in their study (0.9%) was not substantially different from ours (1.4%), and they reported a trend toward higher calcium (adjusted for albumin) in subjects bearing a 986S allele.

TABLE 3. Mean iCa concentrations according to CASR haplotype

Haplotype	n	iCa (mmol/liter)
AGQ/ARQ	27	1.208 ± 0.007[a]
ARQ/ARQ[b]	**180**	**1.220 ± 0.003**
SRQ/ARQ	117	1.235 ± 0.004
SRQ/AGQ	4	1.246 ± 0.030
SRQ/SRQ	27	1.247 ± 0.007
ARE/ARQ	16	1.250 ± 0.011
AGQ/AGQ	1	1.270
SRQ/ARE	5	1.272 ± 0.006

[a] Data are expressed as mean ± SE.
[b] Common haplotypes are in *boldface*.

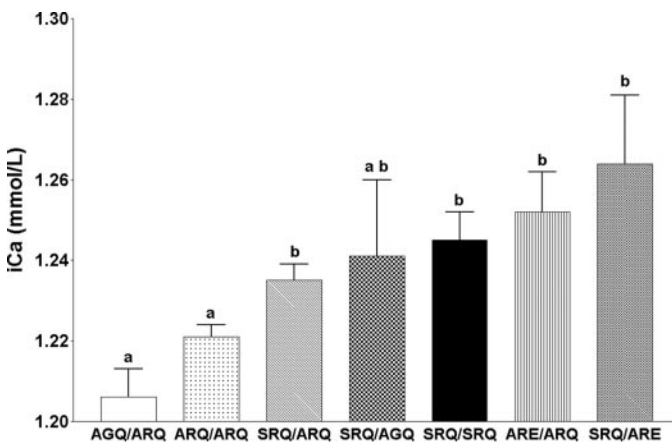

FIG. 1. iCa concentrations according to tri-locus CASR haplotype. Values are adjusted for age, gender/menopausal status, serum creatinine, and PTH using general linear modeling. Groups sharing the *same letter* (*above the error bar*) are not significantly different ($P >= 0.05$).

The present study provides additional evidence for correlation of the ionized calcium phenotype with CASR genotypes. The prevalence of the 990G variant allele was different from that in the North American cohort (4.4% *vs.* 13.2% respectively; $P = 0.0001$), and it was associated with significantly lower iCa in this population, a finding not observed in 44 Japanese subjects (16) with a much higher background prevalence (56.8%). Whether the findings in the Japanese reflect the small sample size is unclear. Although the prevalence of the Q1011E polymorphism was not different from that in the North American cohort (2.8% *vs.* 4.3%, respectively; $P = 0.27$), an isolated 1011E allele was associated with the largest difference in iCa. Whether this relationship will be confirmed in other populations with a higher 1011E prevalence remains to be seen.

Interestingly, the haplotypes were significantly associated with iCa values. In fact, the wild-type genotype (homozygous ARQ) and the genotype bearing the 990G (AGQ) were associated with a lower iCa value, whereas those bearing the 986S (SRQ) and 1011E (ARE) were hypercalcemic. According to these results, the composite genotypes obtained by grouping the four different haplotypes also support a robust association of CASR variants with iCa. In our cohort, we found that iCa decreased in a graded fashion according to genotype, the order being (from highest to lowest): SRQ/ARE>ARE/ARQ>SRQ/SRQ>SRQ/AGQ>SRQ/ARQ>ARQ/ARQ (wild-type)>AGQ/ARQ. Because of methodological differences in the measurement of iCa, direct comparison of different studies is not possible. However, normalization of iCa values allows us to compare trends. In both the European cohort and the Canadian study (Table 4), the AGQ haplotype is associated with modest hypocalcemia, whereas the SRQ haplotype is hypercalcemic and demonstrates a gene dosage effect. Although the associations with the ARE haplotype are discordant, the number of patients with that haplotype in the Canadian cohort is very

TABLE 4. Ionized calcium by haplotype in European and North-American[a] cohorts

	AGQ/ARQ	ARQ/ARQ	SRQ/ARQ	SRQ/SRQ	ARE/ARQ
Toronto	−0.366 ± 1.008[b] (12)	**−0.097 ± 0.805 (30)**	0.218 ± 0.992 (26)	0.276 ± 0.489 (4)	−0.183 ± 0.905 (8)
Apulia	−0.277 ± 0.783 (27)	**−0.072 ± 0.732 (180)**	0.057 ± 0.840 (117)	0.480 ± 0.800 (27)	0.415 ± 0.974 (16)

[a] Haplotypes and iCa concentrations for the North-American cohort derived from the data set previously published (7).
[b] Values [mean ± SD (n)] for iCa transformed to the standard normal function, with mean of zero and SD of one.

small. More relevant, perhaps, are observations by other investigators (10, 12) that also suggest higher serum calcium values associated with the 1011E genotype.

The multiple regression analysis showing the substantial predictive power of the genotypes on calcium (accounting for about half the variance) also confirms the strength of the multilocus association. That the new cohort was comprised of men and women allowed us to ask whether there were gender-specific effects, but we found none.

The functional properties of the three missense variants in the CASR intracellular tail remain unknown. At present, there is little evidence that they are disease-causing, but their potential role in disease predisposition is stimulating considerable interest. On the basis of a CASR polymorphism haplotype study in stone-forming patients, it was suggested that the 990G variant could influence renal CASR activation and calcium excretion (10). Thus, the tri-locus haplotype may be important in predisposition to renal stones in some fraction of individuals with nephrolithiasis. However, in individuals affected by primary hyperparathyroidism (12), osteoporosis (20), or fragility fractures (21), CASR polymorphisms were not associated with clinical phenotype.

In conclusion, our study confirms the association of increased iCa with the 986S variant allele, but also indicates that the two neighboring loci of exon 7 of CASR are significant predictors. This SNP cluster is an interesting candidate for studying the susceptibility of diseases of calcium metabolism. Moreover, given the between-population variations in the prevalence of variant alleles in CASR SNPs, tri-locus haplotyping could be more informative in studies of association between variation in CASR and disease.

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