CLINICAL STUDY

Frequency of the calcium-sensing receptor variant A986S in patients with primary hyperparathyroidism

S Miedlich, P Lamesch¹, A Mueller² and R Paschke

III. Medical Department, ¹II. Surgical Department and ²Institute of Pathology, University of Leipzig, Leipzig, Germany

(Correspondence should be addressed to R Paschke, III. Medical Department, University of Leipzig, Philipp-Rosenthal Strasse 27, 04103 Leipzig, Germany; Email pasr@server3.medizin.uni-leipzig.de)

Abstract

Objective: Recent studies have shown an influence of the calcium-sensing receptor variant A986S on the serum calcium concentration, suggesting that this genetic variant could be a candidate for various bone and mineral disorders. The intention of this study was therefore to investigate the frequency of the described calcium-sensing receptor variants A986S, R990G and Q1011E in patients with primary hyperparathyroidism to test the hypothesis as to whether these variants represent risk factors for the development of primary hyperparathyroidism.

Design: Fifty patients with primary hyperparathyroidism were included in the study. One hundred and two healthy blood donors served as controls.

Methods: Detection of the genetic variants A986S, R990G and Q1011E was done by direct sequencing of exon 7 of the calcium-sensing receptor in leucocyte DNA.

Results: The heterozygous variant A986S was found in 34% (17 of 50) of the healthy age- and sex-matched controls and 40% (20 of 50) of the patients with primary hyperparathyroidism. This difference was not statistically significant ($P = 0.68$). However, in male patients the heterozygous variant A986S was found more frequently (67%, 6 of 9) than in male controls (20%, 2 of 10, $P = 0.07$). The variants R990G and Q1011E were found less frequently (8–20%) in patients and controls without significant differences between the groups. Patients with the heterozygous variant Q1011E had significantly higher serum calcium and parathyroid hormone levels than patients with the wild-type variant ($P < 0.01$). There was no correlation of serum calcium (total and corrected for albumin) with the calcium sensing receptor variant A986S in 102 healthy blood donors ($P = 0.45$).

Conclusions: The calcium-sensing receptor variants do not, therefore, seem to be major genetic determinants for the development of primary hyperparathyroidism. The variant A986S may possibly represent a risk factor for the development of parathyroid neoplasia in men. Moreover, the presence of the genotype Q1011E might influence the clinical course of the disease. The previously reported significant correlation of serum calcium levels with the genetic variant A986S in healthy subjects could not be confirmed.

European Journal of Endocrinology 145 421–427

Introduction

The extracellular calcium concentration is regulated by the calcium-sensing receptor (CaSR) which was cloned in 1993 (1). This receptor belongs to the family of G protein-coupled receptors (family C). Agonists of this subgroup of G protein-coupled receptors are small molecules, e.g. amino acids and ions, which are present in the extracellular compartment. In the parathyroid glands, binding of calcium to the receptor activates phospholipase C and results in an increase of intracellular calcium via accumulation of inositol-1,4,5-phosphate. Furthermore, cyclic AMP is reduced via inhibition of adenylate cyclase. These mechanisms lead to a reduction of parathyroid hormone secretion and subsequent gene expression (for review see 2).

Patients with homozygous inactivating mutations of the CaSR show severe hypercalcemia due to hyperparathyroidism as well as marked hyperplasia of the parathyroids. Heterozygous inactivating mutations of the CaSR cause benign hypocalciuric hypercalcemia. Patients with this disease show a mild hypercalcemia, a low urinary calcium concentration, normal or mildly elevated levels of parathyroid hormone and mild hyperplasia of the parathyroids (2). However, only recently, in individuals of a large kindred with familial hypercalcemia and a heterozygous mutation of the CaSR, more pronounced alterations such as hypercalciuria, a history of kidney stones, and parathyroid adenomas as well as nodular hyperplasia were reported (3). It can therefore be assumed that impaired CaSR

function directly or indirectly affects proliferation of the parathyroid cells.

Inactivating mutations of the CaSR have been found in the extracellular, transmembrane and the intracellular part of the receptor (4). They result in reduced binding of calcium and subsequent changes in signal transduction. Furthermore, it could be shown that a truncating mutation within the intracellular part of the CaSR results in reduced affinity for calcium and co-operativity for calcium-dependent activation (5).

Apart from mutations in the CaSR, several polymorphisms within the intracellular region have been described (4). They lead to non-conservative exchanges of single amino acids (A986S, R990G, Q1011E). In a prospective study of young healthy women, Cole and coworkers (6, 7) found a significant correlation for the genetic variant A986S with the serum calcium concentrations (total and ionised calcium, as well as calcium corrected for albumin). Therefore, the variant A986S was proposed as a determinant of the extracellular calcium concentration. However, it is not known whether this genetic variant could predispose to disorders of mineral and bone metabolism. Since the calcium levels of the subjects with the hetero- and homozygous variants AS and SS respectively had higher calcium levels than the subjects with the wild-type variant AA (6, 7) it could be assumed that the exchange within the carboxy-terminal domain of the calcium-sensing receptor may influence expression and/or activation of the receptor negatively leading to slightly higher serum calcium concentrations. Subsequently, parathyroid hormone secretion dynamics could be altered and parathyroid cell proliferation could proceed more readily. The latter two sequela may be risk factors for the occurrence of somatic mutations and subsequent clonal cell proliferations within the parathyroids leading to primary hyperparathyroidism (pHPT).

The intention of this study was therefore to investigate the frequency of the CaSR variants A986S, R990G and Q1011E in patients with pHPT to test the hypothesis as to whether these genetic variants could contribute to the development of parathyroid neoplasia. Furthermore, we wanted to investigate whether we could confirm the correlation of the serum calcium concentration with the genetic variant A986S as demonstrated by Cole *et al.* (6, 7).

Subjects and methods

Subjects

Fifty patients, all Caucasians, with pHPT (41 females and 9 males, mean age 62.6 ± 9 years) referred consecutively for parathyroidectomy, were included in the study. The morning before the operation blood was collected for determination of total calcium, albumin (kresolphthalein, bromkresolgreen, HITACHI 917; Roche

Diagnostics, GmbH, Mannheim, Germany), ionised calcium (ion-selective electrode; AVL, Mannheim, Germany) and intact parathyroid hormone (IMMULITE intact parathyroid hormone enzyme immunometric assay; Diagnostics Products Corp. (DPC) Biermann, Bad Nauheim, Germany) as well as for extraction of leucocyte DNA.

None of the patients with pHPT had clinical evidence of other endocrinopathies or a family history of any manifestations of multiple endocrine neoplasia type 1 or type 2. A parathyroid adenoma was found in 41 and parathyroid hyperplasia in 9 cases. The diagnosis of parathyroid adenoma or hyperplasia respectively was made by histological analysis. Criteria for a parathyroid adenoma are evidence of a capsule, almost complete absence of fat cells, and normal non-hyperplastic parathyroid tissue at the rim or in other removed parathyroid glands (8).

According to prior statistical power calculations a number of at least 94 subjects would be necessary to detect differences of 0.03 mmol/l in serum calcium concentrations (assuming a standard deviation of 0.1 mmol/l, $\alpha = 0.05$ and $\beta = 0.1$). The mean difference of corrected serum calcium concentrations between subjects with the CaSR variants AA and AS respectively, obtained by Cole *et al.* (6), was approximately 0.07 mmol/l. Therefore, 102 healthy subjects were recruited from blood donors to serve as a control group for testing the correlation of serum calcium with the genetic variant A986S (all Caucasians, 46 females and 56 males, mean ages: females, 54.9 ± 12.4 years; males, 39.5 ± 12.6 years). Blood was obtained for measurement of total calcium and albumin as well as for extraction of DNA. We used total calcium concentrations corrected for the mean albumin concentration of 42.2 g/l for statistical analysis because measurement of ionised calcium under strictly anaerobic conditions which could not always be guaranteed during asservation and transport of the samples to the laboratory.

Fifty age- and sex-matched subjects of the 102 blood donors (40 females, 10 males, mean age 58.3 ± 9 years) served as the control group for comparing the frequencies of the genetic variants with patients with pHPT. Informed consent was obtained from all patients and healthy control subjects participating in the study. The study was approved by the Ethical Committee of the University of Leipzig.

Genetic analysis

Leucocyte DNA was extracted using the QIAamp blood kit (Qiagen, Chatsworth, CA, USA). DNA (100 ng) was amplified in a 50 μ l PCR with the Prime Zyme PCR Kit (Biometra, Göttingen, Germany) and 200 nmol/l of forward and reverse primer according to the manufacturer's protocol. Exon 7 of the CaSR was amplified using the following forward 5'-CAGAAGGTCATCTTTGGCAGCGGCA-3' and reverse

5'-TCTTCCTCAGAGGAAAGGAGTCTGG-3' primers (9). PCR conditions were as follows: initial denaturation at 92 °C for 3 min, 35 cycles at 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 10 min.

Approximately 50 ng of the PCR product was used for direct sequencing with the Applied Biosystems PRISM dye terminator cycle sequencing ready reaction kit (Perkin Elmer Applied Biosystems, Foster City, CA, USA). If a sequence variation was detected, PCR and sequencing reaction were repeated with the DNA for confirmation of the genetic variation.

Statistical analysis

Chi-square test and Fisher's exact test (for allele frequencies of less than five) were used to compare the occurrence of the different allelotypes of the variants A986S, R990G and Q1011E in patients and control subjects. The wild-type variants are referred to as AA (for A986S), RR (for R990G) and QQ (for Q1011E). Heterozygous and homozygous variants are named AS and SS (for A986S), RG and GG (for R990G) and QE and EE (for Q1011E). Furthermore, we applied a multivariate logistic regression analysis to test the predictive value of the variables, age, gender and genotype, to correctly classify subjects as patients (group = 1) or healthy probands (group = 2).

Multivariate linear regression analysis was applied to identify determinants of the serum calcium concentration in the healthy controls ($n = 102$).

Analysis of variance (one-way ANOVA) was performed to test statistical differences between means of three or more groups. Unpaired *t*-tests were used to test differences between two groups.

For statistical analysis by one-way ANOVA, total serum calcium concentrations ($\text{calcium}_{\text{total}}$) of the control subjects ($n = 102$) were corrected for the mean albumin concentration of 42.2 g/l = $[\text{calcium}_{\text{corr}}]$. The equation for correction was calculated by means of a best-fit linear regression for total calcium against albumin. With the line of best fit the correction

equation in our data set was $[\text{calcium}_{\text{corr}}] = [\text{calcium}_{\text{total}}] - \{([\text{albumin}] - 42.2) \times 0.03\}$.

All statistical analyses were done with the statistical package SPSS, version 8.0 (SPSS Inc., Chicago, IL, USA). A *P* value < 0.05 was considered as significant.

Results

The heterozygous and homozygous genetic variant A986S was found in 40% (20 of 50) of the patients with pHPT (AS in 40%, SS in 0%) and in 34% (17 of 50) of the healthy age- and sex-matched subjects (AS in 30%, SS in 4%). Because of the low frequencies of SS (0 and 4% respectively) patients and controls were divided into two groups for further statistical analysis according to the presence or absence of the allele S (AA vs AS and SS). The difference of the allele frequencies in patient vs control group was not statistically significant ($P = 0.68$). The frequencies of the allelotypes AA, AS and SS in all tested healthy subjects ($n = 102$) were 71.5%, 25.5% and 3% respectively. The latter allelotype frequencies showed a trend towards a higher occurrence of the variant A986S in patients with pHPT which is, however, not statistically significant ($P = 0.2$, Table 1).

Moreover, we studied the frequencies of the different allelotypes separately for female and male patients (females, $n = 41$; males, $n = 9$) and controls (females, $n = 40$; males, $n = 10$). Surprisingly, the allelotype A986S was found more often in male patients compared with matched male controls ($P = 0.07$) whereas female patients and controls showed similar distributions of the genetic variants AS and SS ($P = 0.65$). Comparison of the frequencies of the allelotypes AS and SS in male patients ($n = 9$) and all male controls ($n = 56$) confirmed the above result at a significant level ($P = 0.03$). However, the group of male patients with pHPT was too small to draw clear conclusions.

Multivariate logistic regression analysis (dependent variable: group; independent variables: age, sex, genotypes) did not reveal a significant influence of

Table 1 Mean age and relative frequencies of the genetic variant A986S for patients with pHPT and healthy controls. The frequencies of the allelotypes are listed separately for female (patients: $n = 41$, controls: $n = 40$) and male (patients: $n = 9$, controls: $n = 10$) subjects.

	Mean age in years	A986S			
		AA		AS, SS	
		Female	Male	Female	Male
Patients ($n = 50$)	62.6	30 (60%)		20 (40%)	
		27	3	14	6
Age-matched controls ($n = 50$)	58.3	33 (66%)		17 (34%)	
		25	8	15	2
All controls ($n = 102$)	46.2	73 (71.5%)		29 (28.5%)	
		30	43	16	13

Table 2 Clinical data (calcium_{total}, calcium_{ionised}, intact parathyroid hormone (iPTH), age, symptoms) of the patients with and without the genetic variant A986S.

Patient data	A986S	
	AA	AS
Calcium _{total} (mmol/l $\pm$ S.E.M.)	2.96 $\pm$ 0.09	2.92 $\pm$ 0.06
Calcium _{ionised} (mmol/l $\pm$ S.E.M.)	1.57 $\pm$ 0.03	1.55 $\pm$ 0.03
iPTH (pg/ml $\pm$ S.E.M.)	203.3 $\pm$ 55.0	198.5 $\pm$ 45.3
Age (years $\pm$ S.E.M.)	63.5 $\pm$ 1.5	61.4 $\pm$ 2.3
Hypertonus (n.a.p./n.n.p.)*	17/4	7/7
Dyspepsia (n.a.p./n.n.p.)*	12/18	3/11
Nephrolithiasis (n.a.p./n.n.p.)*	12/17	5/9
Osteoporosis (n.a.p./n.n.p.)*	21/3	13/1

n.a.p. = number of affected patients, n.n.p. = number of non-affected patients.

*Clinical data could only be obtained for 38–44 patients. Statistical comparison of the clinical data revealed no statistically significant differences (*P* values between 0.23 and 0.95).

any of the three genetic variants on the variable group (1 = diseased, 2 = healthy) testing all subjects. Only the variable age was included in the regression model. However, testing age- and sex-matched subjects separately showed a significant predictive value of the variable A986S in men (*P* = 0.05), supporting the above-mentioned observation of a higher frequency of the variant A986S in male patients compared with controls.

In patients with pHPT, the genotype A986S was not associated with any clinical parameters (calcium_{total}, calcium_{ionised}, intact parathyroid hormone (iPTH), age, symptoms; Table 2). However, patients with the genotype RG (R990G) were significantly older than patients with the wild-type variant. Patients with the genotype QE vs QQ (Q1011E) showed significantly higher total serum calcium concentrations as well as higher parathyroid hormone levels. Ionised serum calcium levels were, however, not statistically different between the two genotypes (Table 3).

There was no difference in the relative frequencies of the polymorphism A986S in patients with a solitary

adenoma compared with patients with parathyroid hyperplasia (AS in 33% of patients with hyperplasia (3 of 9) and in 41.5% of patients with a solitary adenoma (17 of 41), *P* = 0.6). Furthermore, frequencies of the histological diagnoses for the different genetic variants of R990G and Q1011E were not different either (*P* = 0.09 and 0.16 respectively).

Cole *et al.* (6) reported evidence of linkage disequilibrium for A986S with both adjacent genetic loci R990G (*P* = 0.045) and Q1011E (*P* = 0.028). However, although in our data set there seems to be a tendency towards a linkage disequilibrium between the loci A986S and the adjacent loci R990G or Q1011E this difference was not statistically significant (*P* between 0.14 and 0.7, tested for all individuals and patients and healthy probands separately, Table 4).

Comparison of the frequencies of the genetic variants R990G and Q1011E which occurred less often revealed no significant differences between patients and normal subjects (Table 5). Because of the rare occurrences of the homozygous variants GG (0–6%) and EE (0%) the frequencies of the hetero- and homozygous polymorphisms (RG, GG and QE, EE) were added for statistical analysis (Chi-square and Fisher's exact tests). Whereas, in the control group, the frequencies of the variants QQ, QE and EE were comparable with those reported by Cole and others (6) the frequencies of the allelotypes RR, RG and GG, reported here, were slightly different. Thus, the variants RG and GG occurred less frequently than in the Canadian study (13.7% vs 21.5%).

Furthermore, we tested whether the genotype A986S correlates with total and corrected serum calcium concentrations as recently reported by Cole and others (6, 7). Corrected serum calcium concentrations were calculated for 102 normal subjects. Analysis of the calcium levels in view of the genotype A986S (AA, AS, SS) by one-way ANOVA was not significant (*P* = 0.45, Fig. 1). The calculated mean corrected calcium levels of the subjects with the allelotype AA (*n* = 73) were 2.39 mmol/l, with AS 2.41 mmol/l (*n* =

Table 3 Clinical data (calcium_{ges}, calcium_{ion}, iPTH, age, symptoms) of the patients with and without the genetic variants R990G and Q1011E.

Patient data	R990G		Q1011E	
	RR	RG	QQ	QE
Calcium _{ges} (mmol/l $\pm$ S.E.M.)	2.95 $\pm$ 0.06	2.83 $\pm$ 0.06	2.88 $\pm$ 0.03	3.31 $\pm$ 0.35†
Calcium _{ion} (mmol/l $\pm$ S.E.M.)	1.56 $\pm$ 0.02	1.51 $\pm$ 0.06	1.55 $\pm$ 0.02	1.62 $\pm$ 0.03
iPTH (pg/ml $\pm$ S.E.M.)	192.5 $\pm$ 39.9	163.3 $\pm$ 51.7	147.9 $\pm$ 18.0	453.1 $\pm$ 230.4†
Age (years $\pm$ S.E.M.)	61.7 $\pm$ 1.3	72.5 $\pm$ 4.0*	62.9 $\pm$ 1.5	60.6 $\pm$ 1.5
Hypertonus (n.a.p./n.n.p.)*	19/7	2/2	18/17	3/2
Dyspepsia (n.a.p./n.n.p.)*	13/23	1/3	11/25	3/1
Nephrolithiasis (n.a.p./n.n.p.)*	13/22	1/3	13/22	1/3
Osteoporosis (n.a.p./n.n.p.)*	27/4	3/0	26/4	4/0

n.a.p. = number of affected patients, n.n.p. = number of non-affected patients.

*Clinical data could only be obtained for 34–40 patients.

†*P* < 0.05 (RR vs RG), †*P* < 0.01 (QQ vs QE).

Table 4 Linking analyses of A986S to the adjacent loci R990G and Q1011E, separately tested for patients, age-matched controls and all controls. *P* values ranged between 0.14 and 0.7.

Genotype	Frequency	R990G			Q1011E		
		RR	RG	GG	QQ	QE	EE
Patients							
AA	30	26	4	0	24	6	0
AS	20	20	0	0	19	1	0
SS	0	0	0	0	0	0	0
Age-matched controls							
AA	33	25	5	3	29	4	0
AS	15	13	2	0	14	1	0
SS	2	2	0	0	2	0	0
All controls							
AA	73	61	9	3	65	8	0
AS	26	24	2	0	25	1	0
SS	3	3	0	0	3	0	0

26) and with SS 2.45 mmol/l ($n = 3$), which indicates a tendency towards higher calcium levels in subjects with the genotype AS and SS. However, summarizing the allelotypes AS and SS for analysis with an unpaired *t*-test (AA vs AS and SS) did not show significant differences between the genotypes ($P = 0.26$). Comparison of the total serum calcium concentrations between individuals with different allelotypes of A986S revealed no statistically significant differences either. Calculated separately for men and women respectively, for corrected calcium levels the same tendencies but no significant differences were observed. Although the male controls had significantly higher levels of total serum calcium than the female controls this phenomenon could be observed for all three allelotypes of A986S. This was very likely due to different albumin levels, as they were significantly higher in men and the differences in total serum calcium levels between men and women could not be confirmed for corrected serum calcium concentrations. Vice versa, we tested whether higher calcium levels correlated with a higher frequency of the hetero- or homozygous variant A986S. All subjects were divided into three groups with low ($n = 35$), intermediate ($n = 32$) and high calcium levels ($n = 35$). In the 'low calcium' group, the variants AS and SS were both found in 7 out of 35 people, in the 'high calcium' group this genetic variant was detected in 13 out of 35 people. However, this difference was not statistically significant ($P = 0.11$).

Figure 1 Comparison of the corrected serum calcium concentrations in healthy subjects with the genotypes AA ($n = 73$), AS ($n = 26$) or SS ($n = 3$). Differences of the mean values were not statistically significant (one-way ANOVA). Mean corrected serum calcium concentrations $\pm$ S.D. are shown for the different genotypes.

Moreover, we tested the influence of the variables albumin, age, sex, and the different genotypes A986S, R990G and Q1011E on the serum calcium concentration with a multivariate linear regression analysis. Albumin and age but not the genotypes A986S, R990G or Q1011E were found to influence the serum calcium concentration at a highly significant level ($P < 0.005$, Table 6).

Discussion

The recently described association of the CaSR variant A986S with higher serum calcium levels in healthy women (6, 7) raises the question as to whether this genetic variant could represent a risk factor for the development of diseases of mineral and bone metabolism. The intention of this study was to test the hypothesis as to whether the presence of the genetic variant A986S within the intracellular domain of the calcium-sensing receptor predisposes to benign

Table 5 Relative frequencies of the genetic variants R990G and Q1011E in patients with pHPT and healthy controls.

	R990G		Q1011E	
	RR	RG, GG	QQ	QE, EE
Patients ($n = 50$)	46 (92%)	4 (8%)	43 (86%)	7 (14%)
Age-matched controls ($n = 50$)	40 (80%)	10 (20%)	45 (90%)	5 (10%)
All controls ($n = 102$)	88 (86.3%)	14 (13.7%)	93 (91.2%)	9 (8.8%)

Table 6 Multivariate linear regression analysis for total serum calcium concentration in 102 healthy subjects. Only albumin and age but not sex or genotypes were included in the model.

Variable	t value	Standardized coefficient (β)	P value
Albumin	11.49	0.79	<0.001
Age	2.94	0.21	0.004
Sex	-0.85	-0.07	0.40
A986S	0.77	0.05	0.44
R990G	-1.25	-0.08	0.14
Q1011E	1.51	0.1	0.21

parathyroid neoplasms. The variant A986S was found in 40% of the patients with pHPT and in 34% of the age-matched control group. This difference was not statistically significant. The frequency of this genetic variant in all healthy subjects tested was lower than in the age-matched group (28.5%). Others (6, 7, 10) have found the variant A986S in 29% of healthy women. Therefore, although a trend towards a higher frequency of the variant A986S in patients with pHPT compared with healthy subjects has been observed, this genetic variant does not seem to be a major risk factor for the development of primary parathyroid neoplasia.

Analysis of the allelotype occurrences of A986S for men and women separately reveals a strong trend towards higher frequencies of the genetic variants AS and SS in male patients with pHPT compared with controls (67% vs 20%). This may indicate that the CaSR variant A986S could be a risk factor for the development of parathyroid adenomas in men but not in women. Furthermore, the result suggests a different pathophysiology of pHPT in men and women which would be consistent with the well-known gender differences regarding the incidence of parathyroid neoplasia (11). However, due to the rare occurrence of pHPT in men it will be difficult to further confirm this result.

Analysis of the clinical data of the patients with the receptor variant A986S did not reveal significant differences compared with patients with the wild-type variant. The presence or absence of the allelotype AS therefore does not influence the course or the symptoms of pHPT. However, patients with the genotype RG (R990G) were significantly older, and total serum calcium as well as parathyroid hormone levels were significantly higher in patients with the genotype QE (Q1011E). This may point to an influence of the latter genetic variants on the clinical progression of the disease. It should be noted, however, that these results require further confirmation due to the rare occurrences of the latter genotypes ($n = 4-6$). Others, however, found an association of lower parathyroid hormone levels with the genotypes RG and GG in Japanese patients with pHPT where this genetic variant occurs much more

frequently than in our study group. Different ethnic and genetic backgrounds of the patients investigated may, at least partly, account for the discrepant results.

Are there other genetic variations which could predispose to parathyroid neoplasia? Previous studies investigated genetic polymorphisms within intron 8 (Bb, Aa) and exon 9 (Tt) of the vitamin D receptor (VDR). Presence of the restriction sites was denoted as bat, absence as BAT (12). The incidence of the genotype baT was significantly higher in patients with sporadic pHPT compared with age-matched controls. The authors suggested that the baT genotype could be responsible for a reduced VDR expression or function whereby an impaired antiproliferative effect and reduced inhibition of parathyroid hormone gene transcription could ensue. However, further studies could not reproduce these results (13, 14).

Other studies investigated the role of estrogen receptor or of parathyroid hormone gene variants as risk factors for the development of pHPT. Carling *et al.* (15) could not find a higher incidence of genetic variations within the estrogen receptor gene (P/p, X/x) in patients with pHPT. A recent study investigated the frequency of polymorphisms of the parathyroid hormone gene (B/b, D/d) in patients with pHPT without finding significant differences of the allelotypes between patients and controls (16).

Therefore, there does not seem to be a single genetic polymorphism which clearly represents a major risk factor for the development of pHPT. However, it is possible that the presence of two or more genetic variations such as the VDR polymorphism bb and the CaSR polymorphism AS may indeed increase the risk for the development of pHPT. It is well known that the presence of functioning VDRs and calcium-sensing receptors represent major determinants for parathyroid hormone secretion and cellular proliferation. In parathyroid cells expressing these two genetic variants a slightly altered regulation of parathyroid hormone secretion and susceptibility to proliferative stimuli could ensue. In response to calcium and/or vitamin D deficiency these genetic variants may lead to a more pronounced proliferation within the parathyroids. This may subsequently trigger somatic mutations and clonal cell expansions.

Furthermore, we have investigated the recently described linkage disequilibrium of the genotype A986S to the adjacent loci R990G and Q1011E as well as the association of the CaSR variant A986S with higher serum calcium levels in healthy subjects (6). Although the relative absence of double heterozygotes points to a linkage disequilibrium, this effect could not be confirmed at a significant level. Moreover, whereas the mean values of corrected serum calcium levels were slightly higher in subjects with the genotypes AS or SS, this difference was not statistically significant. Therefore, from this study it cannot be concluded that the A986S polymorphism represents a major genetic

determinant of the extracellular calcium concentration. The discrepant results could be due to several reasons. First, different genetic backgrounds of the groups investigated may have influenced the results. Second, in our study very small differences could be masked by the selection of a relatively heterogeneous control group of male and female subjects of different age groups and, presumably, a different sex steroid status of the women investigated. In particular, the mean age of the women in our group is clearly different from the groups characterized by other authors (6, 7, 10). However, men and women in our control group did not differ significantly with respect to their corrected serum calcium levels. Third, the differences between the corrected serum calcium concentrations of subjects with the variants AA and AS may be too small to be confirmed at a significant level in our study. The overall frequencies of the polymorphism A986S in this study were similar to those reported by others (6, 7, 10). However, in a Japanese population much lower frequencies of the genotypes AS and SS of codon A986S (AS in 3% and SS in 0%) were reported, suggesting influences of a different genetic and ethnic background (17).

In vitro functional characterization of the CaSR variants is underway to gain further insights into its consequences for the regulation of extracellular calcium concentration.

Acknowledgements

We are indebted to J Forberg (Institute for Medical Information Studies, Statistics and Epidemiology, University of Leipzig) for expert assistance with statistical analysis of the data.

References

- Brown EM, Gamba G, Riccardi D, Lombardi M, Butters R, Kifor O *et al.* Cloning and characterization of an extracellular Ca(2+)-sensing receptor from bovine parathyroid. *Nature* 1993 **366** 575–580.
- Brown EM, Pollak M & Hebert SC. The extracellular calcium-sensing receptor: its role in health and disease. *Annual Reviews of Medicine* 1998 **49** 15–29.
- Carling T, Szabo E, Bai M, Ridefelt P, Westin G, Gustavsson P *et al.* Familial hypercalcemia and hypercalciuria caused by a novel mutation in the cytoplasmic tail of the calcium receptor. *Journal of Clinical Endocrinology and Metabolism* 2000 **85** 2042–2047.
- Heath H III, Odelberg S, Jackson CE, Teh BT, Hayward N, Larsson C *et al.* Clustered inactivating mutations and benign polymorphisms in familial benign hypocalciuric hypercalcemia suggest receptor functional domains. *Journal of Clinical Endocrinology and Metabolism* 1996 **81** 1312–1317.
- Gama L & Breitwieser GE. A carboxy-terminal domain controls the cooperativity for extracellular Ca²⁺ activation of the human calcium sensing receptor. A study with receptor-green fluorescent protein fusions. *Journal of Biological Chemistry* 1998 **273** 29712–29718.
- Cole DEC, Peltekova VD, Rubin LA, Hawker GA, Vieth R, Liew CC *et al.* A986S polymorphism of the calcium-sensing receptor and circulating calcium concentrations. *Lancet* 1999 **353** 112–115.
- Cole DE, Vieth R, Trang HM, Wong BY, Hendy GN & Rubin LA. Association between total serum calcium and the A986S polymorphism of the calcium-sensing receptor gene. *Molecular Genetics and Metabolism* 2001 **72** 168–174.
- Black WCI & Utley JR. The differential diagnosis of parathyroid adenoma and chief cell hyperplasia. *American Journal of Clinical Pathology* 1968 **49** 761–775.
- Pollak MR, Brown EM, Estep HL, Chou YH, Hebert SC, Marx SJ *et al.* Mutations in the human Ca²⁺-sensing receptor gene cause familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism. *Cell* 1993 **75** 1297–1303.
- Lorentzon M, Lorentzon R, Lerner UH & Nordstrom P. Calcium sensing receptor gene polymorphism, circulating calcium concentrations and bone mineral density in healthy adolescent girls. *European Journal of Endocrinology* 2001 **144** 257–261.
- Silverberg SJ & Bilezikian JP. Primary hyperparathyroidism: still evolving? *Journal of Bone and Mineral Research* 1997 **12** 856–862.
- Carling T, Kindmark A, Hellman P, Lundgren E, Ljunghall S, Rastad J *et al.* Vitamin D receptor genotypes in primary hyperparathyroidism. *Nature Medicine* 1995 **12** 1309–1311.
- Kobayashi T, Sugimoto T, Kobayashi A & Chihara K. Vitamin D receptor genotype is associated with cortical bone loss in Japanese patients with primary hyperparathyroidism. *Endocrine Journal* 1998 **45** 123–125.
- Menarguez J, Goicoechea M, Crist bal E, Arribas B, Martinez ME, Alcazar JA *et al.* Lack of relationship between BsmI vitamin D receptor polymorphism and primary hyperparathyroidism in a Spanish female population. *Calcified Tissue International* 1999 **65** 214–216.
- Carling T, Rastad J, Kindmark A, Lundgren E, Ljunghall S & Akerström G. Estrogen receptor polymorphism in postmenopausal primary hyperparathyroidism. *Surgery* 1997 **122** 1101–1106.
- Kanzawa M, Sugimoto T, Kobayashi A & Chihara K. Parathyroid hormone gene polymorphisms in primary hyperparathyroidism. *Clinical Endocrinology* 1999 **50** 583–588.
- Kanazawa H, Tanaka H, Shimizu J & Seino Y. A986S polymorphism of calcium-sensing receptor. *Lancet* 1999 **353** 1274.

Received 30 March 2001

Accepted 28 June 2001