Original Paper

The Calcium-Sensing Receptor Gene Polymorphism rs1801725 and Calcium-Related Phenotypes in Hemodialysis Patients

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Key Words

Calcium • Calcium-sensing receptor gene • Cinacalcet • Hemodialysis • Nephrolithiasis • Parathyroid hormone • Phosphorus • Survival • Vitamin D pathway genes

Abstract

Background/Aims: The calcium-sensing receptor gene (CASR) rs1801725 variant is responsible for a non-conservative amino-acid change (A986S) in the calcium-sensing receptor cytoplasmic tail. We hypothesized that rs1801725 polymorphism might be helpful in understanding Ca-related abnormalities in HD patients. **Methods:** In 1215 subjects (245 on cinacalcet), we determined the associations of rs1801725 with secondary hyperparathyroidism (sHPT)-related laboratory parameters, PTH-decreasing effect of cinacalcet hydrochloride, coronary artery disease (CAD), myocardial infarction (MI), nephrolithiasis-related ESRD, and mortality. CASR rs7652589(A<G)_rs1801725(G>T) haplotypes and rs1801725 epistatic interactions with vitamin D signaling pathway genes were examined for associations with selected phenotypes. **Results:** The rs1801725 variant allele showed an increasing independent effect on plasma PTH ($P_{\text{corrected}} = 0.009$). CASR rs7652589_rs1801725 AT haplotype was associated with 1.7-fold higher frequency of PTH levels over 437 pg/mL than the reference haplotype GG ($P = 0.001$). CASR rs7652589_rs1801725 AG haplotype was 1.5-fold more frequent in nephrolithiasis-related ESRD than the GG haplotype ($P = 0.004$). There were no significant associations between rs1801725, CAD, MI, and response to cinacalcet. Variant homozygosity of rs1801725 correlated independently with higher infection-related mortality compared with heterozygosity (HR 7.95,

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95%CI 2.15 – 29.37, $P = 0.003$) and major homozygosity (HR 5.89, 95%CI 1.69 – 20.55, $P = 0.040$). *CASR* rs1801725 did not show epistatic interactions with vitamin D signaling pathway genes concerning tested associations. **Conclusion:** The variant allele of *CASR* rs1801725 solely and together with the variant allele of rs7652589 increases risk of more advanced sHPT. Homozygosity of the rs1801725 variant allele contributes to infection-related mortality in HD patients.

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Introduction

The majority of end-stage renal disease (ESRD) patients demonstrates secondary hyperparathyroidism (sHPT), which enhances with prolongation of renal replacement therapy (RRT) duration [1] and requires parathyroidectomy (PTx) in the most severe cases [2]. Mineral bone disorders, including sHPT, are closely associated with cardiovascular complications and contribute to morbidity and mortality in hemodialysis (HD) subjects [3]. Serum parathyroid hormone (PTH) concentrations and other features of sHPT [4-7], as well as treatment with cinacalcet [7-9], seem to be associated with calcium-sensing receptor gene (*CASR*) single nucleotide polymorphisms (SNPs).

In our study, the *CASR* rs1801725 SNP was selected for genotyping. The rs1801725 SNP encodes a missense variant in the *CASR* exon 7, which leads to a non-conservative amino-acid change (A986S) in the calcium-sensing receptor (CaSR) cytoplasmic tail. CaSR is sensitive to ionized Ca concentration and regulates PTH secretion in parathyroid cells [10]. In 1999, the variant allele of rs1801725 was associated with higher circulating calcium levels in healthy women [11]. Higher calcium concentrations in subjects harboring the variant allele of rs1801725 were reported as associated with decreased sensitivity of the CaSR to calcium due to the production of a less active receptor by the variant allele of rs1801725 [10, 12]. However, the rs1801725 polymorphism was not a predictor of serum calcium levels either in primary HPT [13] or sHPT [7]. Moreover, two functional studies documented the normal activity of CaSR coded by the variant allele of rs1801725 SNP [14, 15]. Therefore, the impact of the variant rs1801725 allele on HPT phenotypes cannot be simply explained by a less active CaSR, and is still open for investigations.

GWAS concerning serum PTH concentrations in European ancestry showed that among genes showing the most influential association with serum PTH levels are rs6127099 upstream of *CYP24A1* that encodes the catabolic enzyme for 1, 25-dihydroxyvitamin D and 25-dihydroxyvitamin D, and rs73186030 near *CASR* [16]. Other *CASR* SNPs, rs7652589 [6] and rs1801725 [7], were individually involved in serum PTH levels in HD patients. It is not clear whether haplotypes of these SNPs are also associated with PTH levels, and whether they interact with vitamin D pathway genes or not.

The *CASR* rs1801725 polymorphism was not associated with the risk of nephrolithiasis development in a Chinese population [17]. In HD patients, rs7652589 SNP correlated with nephrolithiasis-related ESRD without interaction with SNPs of vitamin D signaling pathway genes [6].

The significant associations of *CASR* rs1801725 with calcium-related outcomes (coronary heart disease - CAD, myocardial infarction - MI, hypertension, stroke, osteoarthritis, osteoporosis, and nephrolithiasis) were not found in Genome-Wide Meta-Analysis [18]. However, in angiographically defined CAD patients, the variant allele of *CASR* rs1801725 was reported to be an independent predictor of CAD, MI, all-cause mortality, and cardiovascular mortality [19].

Data mentioned above indicate that *CASR* rs1801725 affecting serum Ca, Mg, P, and PTH, might be involved in Ca-related phenotypes in HD patients solely, with other *CASR* SNPs, or interacting with vitamin D signaling pathway genes.

Calcimimetics, allosteric activators of CaSR, provide an effective means of reducing PTH secretion in HD patients [20]. Studies of the association between *CASR* and the response

to treatment with the calcimimetic agent cinacalcet included several *CASR* SNPs [6-9] and showed that *CASR* R990G (rs1042636) influences the response to calcimimetics in patients with sHPT [9].

Our study aimed to determine the associations of the *CASR* rs1801725 SNP with sHPT-related laboratory parameters, PTH-decreasing effect of treatment with cinacalcet hydrochloride, Ca-related morbidities (CAD, MI, nephrolithiasis-related ESRD) as well as mortality of HD patients. *CASR* rs7652589_rs1801725 haplotypes and *CASR* rs1801725 SNP epistatic interactions with vitamin D signaling pathway genes were also examined for associations with serum PTH levels, PTH-lowering response to cinacalcet, and nephrolithiasis-related ESRD.

Materials and Methods

Patients

ESRD patients whose current method of RRT was intermittent HD were enrolled in the study. HD subjects who had undergone renal transplantation (RTx) but returned to HD treatment after graft loss were excluded as living with renal graft influences parameters related to sHPT [21, 22] and prolongs lifetime on RRT [23].

Patient enrollment began in January 2009 and ended in October 2017. Among the studied group (n = 1215), there was 1017 (83.7%) individuals genotyped for *CASR* rs7652589 SNP and vitamin D signaling pathway gene SNPs in our previous study [6].

The clinical management of patients was decided by an individual physician and not affected by the study. The dietary and pharmacological treatment of all patients was based on a standard of care according to the physician. Guidelines for this treatment based on international recommendations [24, 25]. Nephrologists had been prescribing calcium carbonate or calcium acetate due to hyperphosphataemia without hypercalcemia or due to hypocalcemia. Sevelamer hydrochloride was used in hypercalcaemic/hyperphosphatemic patients. Among vitamin D supplements, alfacalcidol (Alfadiol, GlaxoSmithKline Pharmaceuticals SA, Poland) was the most frequently used. None of the patients was administered intravenous vitamin D analogs.

Patients with serum PTH levels equal to or exceeding 500 pg/mL could receive treatment with cinacalcet hydrochloride (reimbursed by the National Health Fund - NHF). Treatment with cinacalcet (Mimpara, Amgen Europe, Breda, Netherlands) at a daily dose of 30 mg was started in 245 patients with PTH exceeding 500 pg/mL. Among this group, there were 161 subjects analyzed in the previous study concerning *CASR* rs7652589 SNP [6]. Patients who showed serum PTH of 1000 pg/mL or higher before the planned treatment with cinacalcet underwent 99mTc-MIBI scintigraphy. Only those without scintigraphic suspicion of parathyroid gland adenoma qualified for this treatment; the remaining underwent PTx if possible (no clinical contraindications, written informed consent obtained). In the cinacalcet group, serum PTH was checked every four weeks, and cinacalcet doses were increased up to 180 mg per day, if well tolerated, until the PTH concentration decreased at least by 40% of the initial level, which was considered as a satisfactory response to treatment. Patients who did not exhibit sufficiently reduced serum PTH were referred to as non-responders to cinacalcet.

Blood samples for Ca, P, ALP, and PTH were routinely taken from all HD patients at the onset of RRT. Periodical measurements were conducted every 1-3 months for Ca and P and every 6-12 months for ALP and PTH. Differences in the frequency of measurements were dependent on the intrinsic regulations of the respective dialysis centers.

All study subjects were Caucasians of Polish origin.

Patient Data

Demographic and clinical parameters including date of birth, gender, chronic kidney disease causing ESRD and requiring RRT, time of the start of RRT, treatment with peritoneal dialysis before HD initiation, presence of CAD, a history of MI, and PTx were recorded by a review of the medical charts and by personal communications with the nephrologists responsible for the treatment of patients in the dialysis centers. The

patient data concerning CAD, MI, and PTx were re-evaluated every year from the initiation to the end of the enrollment period.

Laboratory parameters included serum concentrations of total Ca, P, and intact PTH, as well as serum activity of total ALP; all serum concentrations were determined using routine laboratory methods. The laboratory data were evaluated in our database every year: the last four results obtained while the patient was in stable condition were collected as long as their number was not limited by a short HD vintage. For analysis, each laboratory parameter was represented as a mean value of up to 4 results obtained during the last period of HD duration. In patients who underwent PTx or were treated with cinacalcet, laboratory data obtained before those treatments were included in the analysis. Analyses of the *CASR* association with serum calcium were also performed using the lowest Ca levels among up to 4 last collected values, if patients were receiving Ca-containing drugs. The *CASR* associations with circulating phosphorus were also explored using the highest P levels among up to 4 previous collected values if patients were taking phosphate-binding drugs.

Additionally, plasma 25(OH)D levels were determined in 245 blindly selected HD patients. Blood samples for 25(OH)D were taken in the winter to avoid differences in sunlight exposure between patients who sunbathed and those who did not. Plasma 25(OH)D concentrations were measured in HD patients who had not been treated with vitamin D or had stopped vitamin D treatment at least three weeks earlier.

In patients treated with cinacalcet, serum PTH concentrations preceding the initiation of cinacalcet administration and those measured 1-2 months after implementation of the maximum cinacalcet dose were collected. However, serum PTH concentrations were obtained no longer than 12 months from the beginning of treatment.

For the survival analysis, the dates of death and kidney transplantation were recorded. The causes of death were categorized as cardiac (reported as myocardial infarction, sudden cardiac death, severe arrhythmias, cardiomyopathies, or cardiac failure), vascular (reported as cerebrovascular events, cerebral stroke, or generalized atherosclerosis), cancer-related, infection-related, or other. The last evaluation of patient outcomes was performed in January 2018.

Genotyping

Genotyping was performed using leukocyte DNA obtained from the blood.

The *CASR* rs1801725 SNP was genotyped in enrolled HD patients. Analysis of *CASR* rs1801725 variant was performed using pre-designed TaqMan SNP Genotyping Assay according to the manufacturer's instructions provided by Applied Biosystems (Applied Biosystems, Foster City, CA).

CASR rs7652589 SNP and vitamin D signaling pathway gene SNPs were genotyped using previously described methods [6, 26] in all HD patients who were not tested for our earlier studies. Genetic testing included the SNPs in the following vitamin D signaling pathway genes: group-specific component protein, *GC* (rs7041, rs1155563, rs2298849), retinoid X receptor alpha, *RXRα* (rs10881578, rs10776909, rs749759), and vitamin D receptor, *VDR* (rs1544410, rs2228570).

Quality control was ensured by running 10% of the samples in duplicate. Only samples providing unequivocal results were taken for analysis.

Distributions of tested polymorphisms were in concordance with the Hardy-Weinberg equilibrium (HWE).

Statistical analysis

The results are presented as percentages for categorical variables or as the median and range (minimum-maximum) for continuous variables because the majority of continuous variables were non-normally distributed as tested by the Shapiro-Wilk test.

Demographic, clinical and laboratory data were compared in HD subjects stratified by *CASR* rs1801725 genotypes. The results were analyzed using inheritance models (dominant, recessive, and additive). Continuous variables were compared using the Mann-Whitney test. For comparison of categorical variables, Chi-square test or Fisher's test was used, as appropriate. A receiver operating characteristic (ROC) curve analysis was applied to create serum PTH cut-off value for PTH as a dichotomous variable.

Departure from HWE was determined by Chi-squared analysis ($P > 0.05$ for equilibrium). Polymorphisms were tested for trends in association using the Chi-square test if two groups were compared

or the Cochran-Armitage test if three groups were compared (P_{trend}). Genotype distributions were compared between the tested groups using the standard Chi-square test (P_{genotype}).

For categorical variables, Odds ratio (OR) and 95% confidence intervals (CIs) for OR were calculated to quantify how strongly the presence or absence of tested allele, genotype or haplotype is associated with the presence or absence of selected phenotypes of HD patients. Chi-square test or Fisher's test was used for statistical evaluation of OR. All probabilities were two-tailed.

To determine the association of *CASR* SNP with serum PTH level among other relevant patient characteristics, we used the primary logistic regression models with subsequent stepwise logistic regression and backward elimination for selection of significant variables among other possible determinants of serum PTH.

Survival probability since the onset of RRT was analyzed concerning *CASR* genotypes. This analysis was also performed using dominant, recessive and additive models of inheritance. The Kaplan-Meier method with the log-rank test was used to estimate significant differences in the cumulative proportion of survival curves characterizing the genotype groups in each model of inheritance. The Cox proportional hazard model was applied to show whether and to which extend the effect of a unit increase in a covariate was multiplicative concerning the hazard rate of death. Cox proportional hazard model was also applied in multivariate analyses assessing the contribution of demographics and clinical measures to mortality.

The previously mentioned statistical analyses were performed using Statistica version 12 (Stat Soft, Inc., Tulsa, OK, USA) and R software, version 3.4.0.

Haplotype frequencies were estimated using the Haploview 4.2 software (<http://www.broad.mit.edu/mpg/haploview/>). Haplotypes were statistically analyzed if their incidence in the examined group was over 1%. Epistatic interactions between *CASR* SNP and vitamin D signaling pathway gene SNPs were analyzed using multifactor dimensionality reduction (MDR) method [27].

Values of $P < 0.05$ were considered significant in the epistatic interaction, multiple regression, and survival probability analyses. Bonferroni corrected P values were deemed to be significant in statistical analyzes involving OR (%CI) calculations.

Ethical approval

The research design was approved by the Institutional Review Board of Poznan University of Medical Sciences, Poland. Informed consent was obtained from all study participants. The study was carried out following the approved guidelines.

Results

Patient characteristics

The analysis of demographic, clinical and laboratory data of HD patients, stratified by genotypes of *CASR* rs1801725, revealed no significant differences in the examined data, including the prevalence of nephrolithiasis-related ESRD, CAD, and MI (Table 1).

An association of CASR rs1801725 SNP with laboratory parameters related to sHPT

Table 2 shows laboratory parameters related to sHPT in HD subjects classified by the *CASR* rs1801725 genotypes. Patients harboring the variant allele of rs1801725 revealed higher PTH levels than homozygotes of the major allele (the dominant inheritance model). According to a ROC curve analysis, a PTH concentration differing patients bearing the variant allele from those being homozygotes of the major allele was shown to be 437 pg/mL (AUC 0.554, SE 0.018, $P = 0.002$). Prevalence of PTH results over 437 pg/mL was about 1.5-fold higher in subjects bearing the variant allele of rs1801725 (Table 3).

Total calcium and phosphorus concentrations, as well as total alkaline phosphatase (ALP) activity, did not present significant differences in models of inheritance (Table 2). When the lowest calcium concentrations and the highest phosphorus levels among the respective last four collected values were taken into account in the analysis, we also did not show significant associations with *CASR* rs1801725 SNP (Table 4).

Table 1. Differences in selected data of hemodialysis patients concerning *CASR* rs1801725 polymorphic variants. ^a Mann Whitney test for quantitative variables or chi-squared test for categorical variables, ^b Fisher's test, ^c Not significant after a Bonferroni correction ($P > 0.004$). Results are presented as median and range (minimum-maximum) or the number of patients presenting indicated parameter with the % of the total of patients tested in each genotype group shown in parentheses. Conversion factors to SI units are as follows: for 25(OH) D - 1 ng/mL = 2.496 nmol/L. Abbreviations: BMI - body mass index, *CASR* - calcium-sensing receptor gene, ESRD - end-stage renal disease, PD - peritoneal dialysis, RRT - renal replacement therapy

Parameter	GG n = 840	GT n = 347	TT n = 28	Mode of inheritance	Odds ratio (95% CI)	P value ^a
Male gender, n = 674	458 (54.5)	200 (57.6)	16 (57.1)	TT+ GT vs GG	1.133 (0.886 - 1.449)	0.319
				TT vs GT+GG	1.072 (0.503 - 2.286)	0.857
				TT vs GG	1.112 (0.520 - 2.380)	0.784
Age at RRT onset, years n = 1215	62.4 (11.8 - 91.7)	60.7 (11.1 - 89.8)	58.9 (26.3 - 84.6)	TT+ GT vs GG		0.042 ^c
				TT vs. GT+GG		0.491
				TT vs. GG		0.367
Diabetic nephropathy, n = 371	250 (29.8)	113 (32.6)	8 (28.6)	TT+ GT vs GG	1.124 (0.865 - 1.461)	0.381
				TT vs GT+GG	0.908 (0.396 - 2.081)	0.820
				TT vs GG	0.944 (0.410 - 2.172)	0.892
Chronic glomerulonephritis, n = 149	94 (11.2)	53 (15.3)	2 (7.1)	TT+ GT vs GG	1.364 (0.954 - 1.950)	0.088
				TT vs GT+GG	0.544 (0.128 - 2.317)	0.565 ^b
				TT vs GG	0.610 (0.143 - 2.613)	0.760 ^b
Hypertensive nephropathy, n = 255	183 (21.8)	66 (19.0)	6 (21.4)	TT+ GT vs GG	0.853 (0.629 - 1.157)	0.307
				TT vs GT+GG	1.027 (0.412 - 2.561)	0.954
				TT vs GG	0.979 (0.391 - 2.451)	0.964
Nephrolithiasis-related ESRD, n = 99	72 (8.6)	25 (7.2)	2 (7.1)	TT+ GT vs GG	0.828 (0.522 - 1.311)	0.420
				TT vs GT+GG	0.864 (0.202 - 3.697)	1.000 ^b
				TT vs GG	0.821 (0.191 - 3.527)	1.000 ^b
RRT duration, years, n = 1215	5.33 (0.008 - 28.30)	5.13 (0.21 - 26.52)	4.03 (0.009 - 14.49)	TT+ GT vs GG		0.716
				TT vs GT+GG		0.045 ^c
				TT vs GG		0.045 ^c
PD as the first method of RRT, n = 31	17 (2.0)	13 (3.7)	1 (3.6)	TT+ GT vs GG	1.877 (0.916 - 3.850)	0.081
				TT vs GT+GG	1.428 (0.188 - 10.860)	0.519 ^b
				TT vs GG	1.793 (0.230 - 13.970)	0.449 ^b
BMI, kg/m ² n = 1215	25.6 (12.8 - 59.2)	25.4 (14.7 - 44.2)	24.5 (19.6 - 36.0)	TT+ GT vs GG		0.386
				TT vs GT+GG		0.970
				TT vs GG		0.956
Patients with coronary artery disease, n = 485	342 (40.7)	133 (38.3)	10 (35.7)	TT+ GT vs GG	0.898 (0.699 - 1.152)	0.396
				TT vs GT+GG	0.833 (0.381 - 1.820)	0.646
				TT vs GG	0.809 (0.369 - 1.774)	0.596
Patients with a history of myocardial infarction, n = 265	181 (21.5)	77 (22.2)	7 (25.0)	TT+ GT vs GG	1.051 (0.784 - 1.409)	0.740
				TT vs GT+GG	1.200 (0.505 - 2.855)	0.679
				TT vs GG	1.214 (0.508 - 2.900)	0.663
Parathyroidectomized patients, n = 29	17 (2.0)	12 (3.5)	0 (0)	TT+ GT vs GG	1.600 (0.757 - 3.385)	0.215
				TT vs GT+GG	0.689 (0.041 - 11.560)	1.000 ^b
				TT vs GG	0.826 (0.048 - 14.070)	1.000 ^b
Patients treated with cinacalcet, n = 245	158 (18.8)	82 (23.6)	5 (17.9)	TT+ GT vs GG	1.304 (0.970 - 1.753)	0.078
				TT vs GT+GG	0.858 (0.323 - 2.280)	0.758
				TT vs GG	0.938 (0.351 - 2.506)	0.899
25-hydroxyvitamin D, ng/mL n = 245	13.8 (3 - 42) n = 174	12.8 (7 - 23.4) n = 67	25.7 (13.6 - 40) n = 4	TT+ GT vs GG		0.603
				TT vs GT+GG		0.066
				TT vs GG		0.079

Table 2. Associations of *CASR* rs1801725 polymorphic variants with plasma concentrations of calcium, phosphorus, and PTH in hemodialysis patients (n = 1215), ^a Mann Whitney test. Results are presented as median and range (minimum-maximum). P values significant after a Bonferroni correction ($P < 0.004$) are indicated using bold font. Conversion factors to SI units are as follows: for alkaline phosphatase - 1 U/L = 0.0167 μ kat/L, for calcium - 1 mg/dL = 0.25 mmol/L, for phosphorus - 1 mg/dL = 0.323 mmol/L, for PTH - 1 pg/mL = 0.1061 pmol/L. Abbreviations: ALP - alkaline phosphatase, *CASR* - calcium sensing receptor gene, PTH - parathyroid hormone

Parameter	GG n = 840	GT n = 347	TT n = 28	Mode of inheritance	P value ^a
Total calcium, mg/dL	8.83 (5.4 - 12.80)	8.94 (6.65 - 11.41)	8.86 (7.04 - 11.70)	TT+ GT vs GG	0.161 ^a
				TT vs. GT+GG	0.970 ^a
				TT vs GG	0.918 ^a
Phosphorus, mg/dL	4.96 (1.95 - 11.27)	5.10 (1.75 - 10.99)	4.88 (3.44 - 7.28)	TT+ GT vs GG	0.135 ^a
				TT vs. GT+GG	0.695 ^a
				TT vs GG	0.596 ^a
PTH, pg/mL	347.5 (7.3 - 3757)	415 (12.9 - 3269.7)	465.3 (80.1 - 1639.5)	TT+ GT vs GG	0.002
				TT vs GT+GG	0.476
				TT vs GG	0.311
Total ALP, U/L	97.8 (15 - 1684)	97 (13.5 - 977.3)	87 (44 - 425.3)	TT+ GT vs GG	0.428
				TT vs GT+GG	0.545
				TT vs GG	0.617

Table 3. *CASR* rs1801725 genotype and allele frequencies in HD patients with serum PTH >437 pg/mL and ≤437 pg/mL. P values significant after a Bonferroni correction ($P < 0.017$) are indicated using bold font. Abbreviations: HD, hemodialysis; HWE, Hardy-Weinberg equilibrium; MAF, minor allele frequency; PTH, parathyroid hormone

<i>CASR</i> rs1801725	HD patients with serum PTH >437 pg/mL (n, frequency) n = 503	HD patients serum PTH ≤ 437 pg/mL (n, frequency) n = 712	Odds ratio (95% CI)	P value
P _{trend} = 0.002, P _{genotype} = 0.007				
GG	323 (64.2)	517 (72.6)	Reference	
GT	166 (33.0)	181 (25.4)	1.468 (1.141 - 1.889)	0.003
TT	14 (2.8)	14 (2.0)	1.601 (0.753 - 3.401)	0.217
TT+GT vs GG	180 (35.8)	195 (27.4)	1.477 (1.156 - 1.889)	0.002
TT vs GT+GG	14 (2.8)	14 (2.0)	1.427 (0.674 - 3.021)	0.350
MAF	(0.19)	(0.15)	1.389 (1.121 - 1.721)	0.003
P for HWE	0.178	0.689		

Table 4. Associations of *CASR* rs1801725 polymorphic variants with the lowest calcium concentrations and the highest phosphorus levels among the respective last four collected values of hemodialysis patients (n = 1215). ^a Mann Whitney test. Results are presented as median and range (minimum-maximum). Conversion factors to SI units are as follows: for calcium – 1 mg/dL = 0.25 mmol/L, for phosphorus – 1 mg/dL = 0.323 mmol/L

Parameter	GG	GT	TT	Mode of inheritance	P value ^a
Minimum total calcium, mg/dL n = 1165	8.32	8.4	8.4	TT+ GT vs GG	0.367 ^a
	(4.64 - 11.5)	(6.1 - 10.74)	(6.5 - 10.2)	TT vs. GT+GG	0.977 ^a
				TT vs GG	0.905 ^a
Maximum phosphorus, mg/dL n = 1211	5.8	6.0	5.7	TT+ GT vs GG	0.085 ^a
	(2.17 - 13.17)	(2.1 - 13.0)	(3.65 - 9.3)	TT vs GT+GG	0.863 ^a
				TT vs GG	0.721 ^a

CASR SNP as an independent determinant of serum PTH level

Gender, age at RRT onset, RRT duration, diabetic nephropathy, serum Ca and P concentrations, serum ALP activity, 25-hydroxyvitamin D, and the variant allele of *CASR* rs1801725 were chosen as possible associates of serum PTH concentration. *CASR* rs1801725 T allele was a significant positive predictor of serum PTH concentration together with RRT duration, Ca and P concentrations, and total ALP activity. Increasing age and diabetic nephropathy showed a decreasing effect on PTH levels (adjusted $P = 0.009$, Table 5).

Response to treatment with cinacalcet

Patients stratified by *CASR* rs1801725 genotypes and analyzed in models of inheritance did not differ significantly in serum PTH at the therapy onset and maximum doses of cinacalcet (Table 6). There was no significant difference in prevalence of responders to cinacalcet concerning the *CASR* rs1801725 polymorphism (Table 7).

CASR haplotypes

CASR rs7652589_rs1801725 AT haplotype (composed of two variant alleles) was significantly more prevalent ($P_{\text{corr}} = 0.021$) in HD patients with serum PTH over 437 pg/mL and associated with about 1.7-fold higher frequency of plasma PTH values over 437 pg/mL than reference haplotype composed of two major alleles. *CASR* rs7652589_rs1801725 AG haplotype was 1.5-fold more frequent in nephrolithiasis-related ESRD than rs7652589_rs1801725 GG haplotype ($P = 0.004$) (Table 8).

CASR haplotypes were not associated with response to treatment with cinacalcet (Table 9).

Table 5. Associates of serum PTH concentration in HD patients (n = 1215). a – β coefficient and SE values can be interpreted as follows: for a unitary change in an analyzed parameter, serum PTH concentration would change by $\beta \pm$ SE, pg/mL. b – adjustment for variables significant in univariate analyses. c – n = 245. Abbreviations: ALP – alkaline phosphatase, *CASR* – calcium-sensing receptor gene, HD – hemodialysis, PTH – parathyroid hormone, RRT – renal replacement therapy

Parameter	$\beta \pm$ SE ^a	P value	Adjusted P value ^b
Male gender	-38.6 $\pm$ 29.5	0.191	0.595
Age at RRT onset (per 10 years)	-88.5 $\pm$ 9.1	2.0E-21	0.0002
RRT duration (per 1 year)	36.6 $\pm$ 3.4	1.5E-26	2.3E-13
Diabetic nephropathy	-134.4 $\pm$ 31.6	2.3E-5	0.0001
Ca (per 1 mg/dL)	61.5 $\pm$ 18.8	0.001	0.005
P (per 1 mg/dL)	77.3 $\pm$ 9.8	6.2E-15	$\approx$ 0
ALP (per 1 U/L)	1.86 $\pm$ 0.11	$\approx$ 0	$\approx$ 0
25-hydroxyvitamin D (per 1 ng/mL) ^c	- 4.57 $\pm$ 5.34	0.393	0.935
<i>CASR</i> rs1801725 T allele	41.3 $\pm$ 15.8	0.009	0.009

Table 6. Effects of treatment with cinacalcet concerning *CASR* rs1801725 polymorphic variants in hemodialysis patients. Results are presented as median and range (minimum-maximum) or number (percentage). a – Mann-Whitney U test for quantitative variables or Chi-squared test for qualitative variables. b – Fisher's test

Parameter	GG n = 158	<i>CASR</i> rs1801725 GT n = 82	TT n = 5	Model of inheritance	P value ^a
Maximum dose of cinacalcet, mg/day	60 (30 - 150)	60 (30 - 180)	60 (30 - 90)	TT + GT vs GG TT vs GT + GG TT vs GG	0.576 0.826 0.781
Parathyroid hormone at the therapy onset, pg/mL	948 (505 - 3280)	957.6 (510.4 - 2991.5)	1146.5 (550 - 1559)	TT + GT vs GG TT vs GT + GG TT vs GG	0.452 0.836 0.725
Parathyroid hormone at 1-2 months after the application of the maximum dose, pg/mL	439.3 (27.1 - 3281)	395.6 (16.2 - 2495.2)	318 (157 - 1059)	TT + GT vs GG TT vs GT + GG TT vs GG	0.727 0.823 0.806
Percentual decrease of the initial parathyroid hormone concentration	53.7 (-122.2 - 96.7)	55.2 (-113.4 - 97.8)	56 (7.6 - 89.9)	TT + GT vs GG TT vs GT + GG TT vs GG	0.388 0.947 0.866
Response to cinacalcet, n, % of cases	115 (72.8)	63 (76.8)	4 (80.0)	TT + GT vs GG TT vs GT + GG TT vs GG	0.469 1.000 ^b 1.000 ^b

Table 7. *CASR* rs1801725 genotype and allele frequencies in HD patients stratified by responsiveness to treatment with cinacalcet. a – Pearson's Chi-squared test, b – Cochran-Armitage Trend Test, c – Fisher's test. Abbreviations: HD, hemodialysis; HWE, Hardy-Weinberg equilibrium; MAF, minor (variant) allele frequency

<i>CASR</i> rs1801725	Non-responders to cinacalcet (n, frequency) n = 63	Responders to cinacalcet (n, frequency) n = 182	Odds ratio (95% CI)	P value ^a
GG	43 (68.3)	115 (63.2)	Reference	
GT	19 (30.2)	63 (34.6)	0.807 (0.433 - 1.501)	0.497
TT	1 (1.6)	4 (2.2)	0.669 (0.073 - 6.151)	1.000 ^b
TT+GT vs GG	20 (31.7)	67 (36.8)	0.798 (0.434 - 1.469)	0.469
TT vs GT+GG	1 (1.6)	4 (2.2)	0.718 (0.079 - 6.545)	1.000 ^b
MAF	(0.17)	(0.20)	0.825 (0.483 - 1.410)	0.482
P for HWE	0.496	0.167		

Table 8. *CASR* rs7652589_rs1801725 haplotypes and prevalence of plasma PTH levels over 437 pg/mL and nephrolithiasis-related ESRD. ^a P value calculated using permutation test and a total of 1,000 permutations, ^b The most common haplotype was used as the reference, ^c All other haplotypes pooled together were used as the reference. Bold font indicates P values significant after correction; OR (95%CI) were considered significant only if their P values were < 0.05 and simultaneously associated with significant P values calculated using permutation test and a total of 1,000 permutations

Haplotype	Freq.	Case,Control Frequencies	Chi Square	P Value	P _{corr} Value ^a	OR (95%CI); p value ^b	OR (95%CI); p value ^c
Plasma parathyroid hormone levels over 437 pg/mL							
GG	0.525	0.486, 0.553	10.564	0.0012	0.0020	reference	0.766 (0.651 - 0.900); 0.0012
AG	0.309	0.321, 0.300	1.191	0.2750	0.5510	1.215 (1.011 - 1.459); 0.0376	1.101 (0.925 - 1.310); 0.2811
GT	0.092	0.103, 0.085	2.254	0.1333	0.2910	1.370 (1.029 - 1.824); 0.0305	1.228 (0.932 - 1.619); 0.1439
AT	0.074	0.090, 0.062	6.868	0.0088	0.0210	1.664 (1.216 - 2.278); 0.0014	1.510 (1.113 - 2.048); 0.0077
Nephrolithiasis-related ESRD							
GG	0.525	0.455, 0.531	4.269	0.0388	0.0730	reference	0.735 (0.549 - 0.984); 0.0380
AG	0.309	0.399, 0.301	8.115	0.0044	0.0070	1.549 (1.129 - 2.126); 0.0064	1.541 (1.143 - 2.077); 0.0043
GT	0.092	0.075, 0.094	0.726	0.3942	0.7400	0.946 (0.537 - 1.666); 0.8469	0.793 (0.460 - 1.369); 0.4045
AT	0.074	0.071, 0.074	0.021	0.8844	0.9980	1.118 (0.622 - 2.010); 0.7088	0.953 (0.541 - 1.679); 0.8681

Table 9. Haplotypes of *CASR* rs7652589_rs1801725 and responsiveness to treatment with cinacalcet in HD patients. ^a p-value calculated using permutation test and a total of 1,000 permutations

SNPs	Haplotype	Freq.	Case,Control Frequencies	Chi Square	P Value	P _{corr} Value ^a
rs7652589_rs1801725	GG	0.503	0.517, 0.460	1.228	0.2677	0.5690
	AG	0.310	0.287, 0.373	3.214	0.0730	0.1530
	GT	0.110	0.120, 0.080	1.567	0.2107	0.4590
	AT	0.078	0.075, 0.087	0.187	0.6651	0.9510

Epistatic interactions between CASR rs1801725 and vitamin D signaling pathway genes

Epistatic interactions between the *CASR* rs1801725 SNP and vitamin D signaling pathway gene SNPs were examined in HD patients with vs. without serum PTH concentration over 437 pg/mL, responsiveness vs. unresponsiveness to treatment with cinacalcet, and nephrolithiasis-related ESRD vs. other causes of ESRD. There were no significant interactions between *CASR* rs1801725 and vitamin D signaling pathway gene SNPs concerning tested phenotypes (Tables 10-12).

Association of the CASR rs1801725 SNP with clinical outcomes of HD patients

A total of 706 HD patients died, and 161 underwent RTx between January 2nd, 2009 and January 25th, 2018.

Using the Kaplan-Meier curve estimate in the entire cohort of HD patients, *CASR* rs1801725 genotypes did not show an effect on all-cause (log-rank test $P = 0.395$), cardiovascular (log-rank test $P = 0.594$), neoplasm-related (log-rank test $P = 0.543$), and infection-related (log-rank test $P = 0.083$) mortality. However, Cox proportional hazard model showed that homozygotes of the variant allele of *CASR* rs1801725 SNP had higher infection-related mortality compared with heterozygotes and homozygotes of the major allele (Figures 1-4). Homozygosity of the *CASR* rs1801725 variant allele was associated with infection-related mortality in the recessive ($P = 0.003$, Fig. 3) and additive ($P = 0.005$, Fig. 4) models of inheritance. If gender, age at RRT onset, diabetic nephropathy, and CAD/MI in the course of RRT were included in the model, only age and *CASR* rs1801725 SNP were associated with infection-related mortality (Table 13).

Table 10. Epistatic interactions between the *CASR* rs1801725 SNP and vitamin D signaling pathway gene SNPs in HD patients with vs. without serum PTH concentration over 437 pg/mL. a - Significance of accuracy, empirical P value based on 1,000 permutations

Genes and rs numbers	Testing Balanced Accuracy	Cross Validation Consistency	p value ^a
<i>GC</i> _rs7041, <i>CASR</i> _rs1801725	0.5125	6/10	0.7380
<i>VDR</i> _rs2228570, <i>RXRA</i> _rs10776909, <i>GC</i> _rs7041	0.5330	5/10	0.5410
<i>VDR</i> _rs2228570, <i>RXRA</i> _rs10776909, <i>GC</i> _rs7041, <i>CASR</i> _rs1801725	0.4747	3/10	0.9700

Table 11. Epistatic interactions between the *CASR* rs1801725 SNP and vitamin D signaling pathway gene SNPs in HD patients with responsiveness vs. unresponsiveness to treatment with cinacalcet. a - Significance of accuracy, empirical P value based on 1,000 permutations

Genes and rs numbers	Testing Balanced Accuracy	Cross Validation Consistency	P value ^a
<i>CASR</i> _rs7652589, <i>RXRA</i> _rs10776909	0.4860	4/10	0.9160
<i>VDR</i> _rs2228570, <i>VDR</i> _rs1544410, <i>GC</i> _rs2298849	0.5290	5/10	0.6700
<i>CASR</i> _rs7652589, <i>VDR</i> _rs2228570, <i>VDR</i> _rs1544410, <i>GC</i> _rs7041	0.5275	6/10	0.6830

Table 12. Epistatic interactions between the *CASR* rs1801725 SNP and vitamin D signaling pathway gene SNPs in HD patients with nephrolithiasis-related ESRD vs. other causes of ESRD. a - Significance of accuracy, empirical P value based on 1,000 permutations

Genes and rs numbers	Testing Balanced Accuracy	Cross Validation Consistency	P value ^a
<i>VDR</i> _rs1544410, <i>GC</i> _rs2298849	0.5010	5/10	0.8250
<i>VDR</i> _rs1544410, <i>GC</i> _rs2298849, <i>CASR</i> _rs1801725	0.4767	4/10	0.9660
<i>VDR</i> _rs2228570, <i>VDR</i> _rs1544410, <i>RXRA</i> _rs10881578, <i>GC</i> _rs7041	0.4464	4/10	0.9980

Fig. 1. The Kaplan-Meier survival analysis of infection-related mortality in the studied hemodialysis patients stratified by *CASR* rs7652589 genotypes. log-rank test P = 0.083; TT vs. GT: HR 7.95, 95%CI 2.15 - 29.37, P = 0.003; TT vs. GG: HR 5.89, 95%CI 1.69 - 20.55, P = 0.040.

Fig. 2. The Kaplan-Meier survival analysis of infection-related mortality in the studied hemodialysis patients stratified by *CASR* rs1801725 genotypes in the dominant model of inheritance. log-rank test $P = 0.508$; Hazard ratio (HR) for death in TT + GT genotype group vs. GG genotype group: HR 0.85, 95%CI 0.51 - 1.41, $P = 0.521$.

Fig. 3. The Kaplan-Meier survival analysis of infection-related mortality in the studied hemodialysis patients stratified by *CASR* rs1801725 genotypes in the recessive model of inheritance. log-rank test $P = 0.137$; Hazard ratio (HR) for death in TT genotype group vs. GG+GT genotype group: HR 6.39, 95%CI 1.85 - 22.14, $P = 0.003$.

Fig. 4. The Kaplan-Meier survival analysis of infection-related mortality in the studied hemodialysis patients stratified by *CASR* rs1801725 genotypes in the additive model of inheritance. log-rank test $P = 0.138$; Hazard ratio (HR) for death in TT genotype group vs. GG genotype group: HR 6.09, 95%CI 1.71 - 21.74, $P = 0.005$.

Table 13. Results of multivariate analyses using Cox proportional hazard model for prediction of variables explaining infection-related mortality in HD patients. a – Hazard ratio for this variable is calculated for an increase of age at RRT onset by one year. Abbreviations: CAD – coronary artery disease, *CASR* – calcium-sensing receptor gene, HD – hemodialysis, MI – myocardial infarction, RRT – renal replacement therapy

Parameters	Gender, age at RRT onset, diabetic nephropathy, CAD, and <i>CASR</i> rs1801725	Gender, age at RRT onset, diabetic nephropathy, MI, and <i>CASR</i> rs1801725
	polymorphic variants	polymorphic variants
Significant predictors	HR, 95% CI, P value	HR, 95% CI, P value
Age at RRT onset ^a	1.03, 1.00 – 1.05, 0.047	1.03, 1.01 – 1.05, 0.011
<i>CASR</i> rs1801725 GG vs. TT	0.16, 0.04 – 0.58, 0.028	0.16, 0.04 – 0.60, 0.026
<i>CASR</i> rs1801725 GT vs. TT	0.13, 0.03 – 0.51, 0.008	0.14, 0.03 – 0.55, 0.012
Age at RRT onset ^a		
<i>CASR</i> rs1801725 GG vs. GT+TT	1.02, 1.01 – 1.05, 0.012	1.03, 1.01 – 1.05, 0.004
(dominant model of inheritance)	NS	NS
Age at RRT onset ^a		
<i>CASR</i> rs1801725 GG + GT vs. TT	1.03, 1.01 – 1.05, 0.005	1.03, 1.01 – 1.05, 0.006
(recessive model of inheritance)	0.16, 0.04 – 0.56, 0.005	0.16, 0.04 – 0.59, 0.006
<i>CASR</i> rs1801725 GG vs. TT		
(additive model of inheritance)	0.12, 0.03 – 0.46, 0.002	0.12, 0.03 – 0.48, 0.003

Discussion

In this study, we have shown that HD patients of European ancestry, who harbor the variant allele of *CASR* rs1801725, develop higher PTH levels than homozygotes of the major allele; therefore, they are prone to more severe sHPT. In this aspect, our study confirms data obtained by Moe et al. [7]. The variant allele of rs1801725 was an independent predictor of serum PTH levels among variables already associated with PTH concentrations in HD patients [1, 28-30]. Our previous study [6] demonstrated that the variant homozygosity of *CASR* rs7652589 was associated with PTH levels over 500 pg/mL. The present data recognize *CASR* rs7652589_rs1801725 AT haplotype (composed of two variant alleles) as associated with more severe sHPT, and indicates that both *CASR* SNPs act in cooperation concerning serum PTH.

Calcimimetics increase CaSR expression [31] and are used to reduce PTH secretion by parathyroid cells in sHPT patients [20]. A decrease in serum PTH concentration in response

to administration of cinacalcet varies between HD patients, but there is no definite evidence showing that *CASR* SNPs have a direct impact on PTH-lowering response to cinacalcet, although several *CASR* SNPs were investigated for association [6-9]. In HD group receiving cinacalcet, we could not show any association between a decrease in serum PTH in response to cinacalcet and *CASR* rs1801725 SNP or *CASR* rs7652589_rs1801725 haplotypes.

In non-uremic subjects, *CASR* rs1801725 SNP was found as attributed to the development of calcium nephrolithiasis [32, 33] and simultaneously higher serum calcium levels [33] or not associated with calcium kidney stone formation [17, 34, 35]. In HD patients, *CASR* rs1801725 SNP did not associate solely either with nephrolithiasis-related ESRD or serum calcium concentration. Therefore, higher frequency of *CASR* rs7652589_rs1801725 AG haplotype in patients showing nephrolithiasis-related ESRD seems to be dependent on the rs7652589 A allele.

Although the variant allele of *CASR* rs1801725 appeared as associated with more advanced sHPT, it was not attributed in this retrospective study to CAD, MI, and all-cause or cardiovascular mortality of HD patients of European ancestry. In the 7-year prospective study, *CASR* rs7652589 was not associated with all-cause and cardiovascular mortality in HD subjects [26]. *CASR* polymorphic variants also had no impact on the occurrence of cardiovascular events and all-cause mortality in renal transplant recipients [36]. Similarly, bone fractures were not attributed to *CASR* rs1801725 [7]. Serum PTH concentration ≥ 600 mg/mL determined mortality of HD patients [30, 37]. The variant allele in *CASR* rs1801725 was associated with serum PTH concentrations over 437 pg/mL. Therefore, PTH levels associated with this variant allele could be too low to separate HD survivors and non-survivors concerning serum PTH.

Homozygosity of the variant allele in *CASR* rs1801725 contributed to higher infection-related mortality of HD patients. Involvement of CaSR or its activators in infectious diseases was suggested in several studies [38, 39]. Human peripheral blood monocytes possess both CaR protein and mRNA [40]. *CASR* rs7652589 SNP interacted with *CCL2* rs1024611 SNP concerning nephrolithiasis-related ESRD which was complicated with near 39% of cases with chronic infective tubulointerstitial nephritis [6]. In the 7-year prospective study, *CASR* rs7652589 yielded a borderline significance concerning infection-related mortality in the dominant model of inheritance [26]. However, how *CASR* rs1801725 SNP influences outcomes of infectious diseases remains unknown.

Limitations of the study

Associations of *CASR* rs1801725 with selected phenotypes were analyzed under conditions of conventional treatment targeting sHPT. Ca-containing drugs were administered the most commonly, serving as phosphate binders and Ca supplements. These drugs were usually started still before HD therapy, and their discontinuation was not possible due to ethical reasons. Although about a half of HD subjects is noncompliant with medication taking [41], Ca-containing drugs and vitamin D supplements could abolish differences in serum Ca and P resulting from genetic variability.

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Disclosure Statement

The authors declare no competing financial interests.

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