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Alanine to Serine Variant at Position 986 of Calcium Sensing Receptor and Colorectal Cancer Risk

Reza Dabiri,[1] Touraj Mahmoudi,[2,*] Hamid Farahani,[3] Hossein Nobakht,[1] and Mohammad Reza Zali[2]

[1]Internal Medicine Department, Semnan University of Medical Sciences, Semnan, IR Iran
[2]Gastroenterology and Liver Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, IR Iran
[3]Department of Physiology, School of Medicine, Qom University of Medical Sciences, Qom, IR Iran

[*]*Corresponding author*: Touraj Mahmoudi, Gastroenterology and Liver Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, IR Iran. Tel: +98-9124148851, Fax: +98-2122432517, E-mail: mahmouditouraj@gmail.com

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Abstract

Background: With regard to the effect of calcium against colorectal cancer (CRC) and considering the key role of calcium sensing receptor (CaSR) in calcium homeostasis, this study investigated whether *CaSR* gene rs1801725 or A986S variant was associated with susceptibility to CRC risk.
Methods: This study was conducted as a case-control study and 303 cases with CRC and 354 controls were enrolled. All 657 subjects were genotyped for *CaSR* gene A986S variant using PCR-RFLP method.
Results: No significant difference was observed for the A986S variant of *CaSR* gene in either genotype or allele frequencies between the cases and the controls and this lack of difference remained non-significant even after adjustment for age, BMI, sex, smoking status, and family history of CRC. No evidence for the effect modification of the association A986S variant and CRC by BMI, sex, or tumor site was also observed. Furthermore, the risk of obesity in relation to the A986S variant in the controls and the cases was separately analyzed and we observed no significant difference between normal weight (BMI $< 25 kg/m^2$) and overweight/obese (BMI $\geq 25 kg/m^2$) subjects.
Conclusions: Our findings do not support a role for effect of the *CaSR* gene A986S variant on CRC risk; nevertheless, this finding requires confirmation and the role of the gene variant in carcinogenesis needs to be further investigated.

Keywords: Calcium Sensing Receptor Gene, Colorectal Cancer, RFLP, Variant

1. Background

Colorectal cancer (CRC) is a major health problem and is the second leading cause of cancer-related mortality (1, 2). Previous studies have demonstrated that high calcium intake may lower the risk of colon cancer (3, 4). On the other hand, calcium sensing receptor (CaSR) which contains seven exons and belongs to the G-protein coupled receptor superfamily has a critical role in the maintenance of calcium homeostasis by modulating PTH secretion from the parathyroid glands (5). The CaSR is expressed in human colonic epithelium and colon cancer cells (6). Calcium and CaSR are well-organized controllers of colonocytes (7) and calcium by signaling through CaSR suppresses normal colonocyte proliferation. Furthermore, calcium stimulates the expression of the tumor suppressor gene E-cadherin via CaSR in colon cancer cells (8). Dysregulation of E-cadherin expression seems to be involved in progression from adenoma to carcinoma (9). Furthermore, activation of CaSR suppresses β-catenin-TCF-4 oncogenic pathway (8).

The relationship between *CaSR* gene polymorphisms and CRC risk has lately garnered a great deal of attention and association studies of *CaSR* gene A986S (rs1801725) variant have been performed to investigate its implication with CRC risk, but the results are somewhat contradictory (10-14) and the role of this gene in the etiology of CRC is still equivocal. Some studies (10, 11, 13, 14) have found no association, other investigations (12) have reported significant associations between the A986S variant and the risk of CRC.

Accordingly, these observations led we look for the possible association of the A986S (rs1801725) variant located in exon 7 of *CaSR* gene with CRC risk. Selection criteria for this polymorphism were based on (a) its use in previous genetic epidemiology studies (b) degree of heterozygosity (c) position in the gene (d), and functional importance.

2. Methods

2.1. Participants

The study population consisted of 303 cases with CRC and 354 controls reporting to the research institute for gastroenterology and liver diseases (RCGLD), Shahid Beheshti University of Medical Sciences. This study was conducted as a case-control study and all the subjects were recruited from patients who were under going colonoscopy either because of various gastrointestinal symptoms or because of they were considered high risk for CRC. Cases were the patient with positive pathologic report for CRC, and eligibility criteria for control subjects included no individual history of colorectal malignancy or polyps (including adenomatous and other polyps). All patients and controls were Iranian and genetically unrelated. Before subject's colonoscopy, self administration had been used to collect the information about their demographic, anthropometric, and clinical characteristics. The present study was approved by the ethical committee of the institute and all study participants were informed about the aims of the study and gave written consents. Body mass index (BMI) of each subject was calculated as body weight divided by height squared (kg/m^2).

2.2. Genotype Analysis

Blood samples from all the subjects were collected in tubes containing EDTA as an anticoagulant and store at 4°C. Genomic DNA was purified from peripheral blood leucocytes using standard "phenol chloroform" method. In this study, genotyping was done by using PCR-RFLP method. Also, genotyping of the *CaSR* gene was performed by investigators who were blinded to the participants' clinical data. The A986S (rs1801725) variant was evaluated by a PCR that amplified a 269 bp fragment using a forward (5'-CTGAGCTTTGATGAGCCTCAGAAGGAC-3') and a reverse (5'-CACTGATGACAAGCTCTGTGAACTGGA-3') primer (15). The PCR reaction was run at 93°C for 10 minutes followed by 35 cycles of 93°C for 45s, 63°C for 30s, 72°C for 45 seconds, and final extension at 72°C for 10 minutes. The PCR products were digested overnight with restriction enzyme Hin1I (Fermentas, Leon-Rot, Germany) at 37°C and the RFLP products were run on 3% agarose gel, and stained with ethidium bromide for visualization under UV light. *CaSR* genotypes of each subject were identified according to the digestion pattern and alleles according to the absence ("T") or presence ("G") of the Hin1I site. Hin1I digestion reveals genotypes denoted TT (269 bp), GT (269, 241 bp and 28 bp), or GG (241 bp and 28 bp). The concordance of genotyping was confirmed by duplicate analysis of approximately 10% of the samples and DNA sequencing of approximately 3% of the samples that all of them were selected randomly (all results were accurate).

2.3. Statistical Methods

We calculated differences in demographic or anthropometric factors using t-test or χ^2 test when appropriate. Testing Hardy-Weinberg equilibrium for the *CaSR* gene A986S variant among cases and controls, separately, and comparisons of the distribution of the allele frequencies between the groups were performed using the χ^2 test. Comparisons of the distribution of the genotype frequencies between the different groups were performed using the logistic regression. Logistic regression analysis was also used to adjust for confounders such as age, BMI, sex, smoking status, and family history of CRC. The odds ratios (OR) are given with the respective 95% confidence intervals (95% CI). SPSS statistical package (version 15.0; SPSS Inc. Chicago, IL, USA) was used to analyze the data. In all statistical tests, a P value of less than 0.05 was considered to indicate statistical significance.

3. Results

3.1. Clinicopathological Analysis

Selected characteristics of the study population and their statistical significance are summarized in Table 1. On average, cases with CRC were older ($P < 0.001$) and less likely to use NSAIDs ($P < 0.001$) when compared with their control counterparts. However, there were no significant differences between the cases with CRC and the controls in terms of BMI, sex, smoking status, and family history of colorectal cancer.

3.2. CaSR Gene A986S Variant Analysis

The distribution of genotypes and alleles of the *CaSR* gene A986S variant in cases with CRC and controls are provided in Table 2. None of the genotype frequency distributions for the A986S variant deviated significantly from the Hardy-Weinberg equilibrium in both cases and controls, suggesting that the alleles are in equilibrium ($P > 0.05$). As shown in Table 2, no significant difference was observed in genotype and allele frequencies between the cases with CRC and the controls for the A986S polymorphism. Furthermore, after adjustment for age, BMI, sex, smoking status and family history of CRC, no significant association between the polymorphism and the risk of CRC was found.

Additionally, when we stratified the analyses by tumor site (Table 3) or sex (Table 4), we found no statistically significant differences in the *CaSR* gene A986S variant either before or after adjustment for confounding factors. We also conducted a breakdown comparison between cases

Table 1. Selected Characteristics of the Study Subjects[a]

Variables	Controls (N = 354)	Cases (N = 303)	P Value
Age, y	43.9 (16.7)	55.7 (12.5)	< 0.001
BMI, kg/m^2	25.0 (4.0)	25.4 (4.7)	0.216
Gender			
Male	181 (51.1)	165 (54.5)	
Female	173 (48.9)	138 (45.5)	0.395
Smoking history			
No	294 (83.0)	247 (81.5)	
Former	48 (13.6)	37 (12.2)	
Current	12 (3.4)	19 (6.3)	0.207
Regular NSAID use			
No	283 (79.9)	289 (95.4)	
Yes	71 (20.1)	14 (4.6)	< 0.001
Family history of colorectal cancer			
No	308 (87.0)	264 (87.1)	
Yes	46 (13.0)	39 (12.9)	0.963
Tumor site			
Colon	-	195 (64.4)	
Rectal	-	108 (35.6)	-
Metastasis			
No	-	276 (91.1)	
Yes	-	27 (8.9)	-
HNPCC			
No	-	282 (93.1)	
Yes	-	21 (6.9)	-

[a]Variables presented as mean (SD) or No. (%).

and controls within different BMI categories (Table 5). In the comparison between normal weight controls and normal weight cases with CRC, as well as in the comparison between overweight/obese controls and overweight/obese cases with CRC, we found no differences between these groups with respect to allele and genotype frequencies of the A986S variant either before or after adjustment for age, sex, smoking status, and family history of CRC.

Finally, in this study the risk of obesity in relation to the *CaSR* gene A986S variant was also examined (data not shown). We observed no significant difference in genotype and allele frequencies between the normal weight cases with CRC and overweight/obese cases with CRC and between normal weight controls and overweight/obese controls for the A986S variant.

4. Discussion

We conducted a case-control study to explore the possible association between the *CaSR* gene A986S (rs1801725) variant and CRC risk. In the present study, no statistically significant difference was found for this variant in either genotype or allele frequencies between the cases with CRC and the controls and this lack of difference remained non-significant even after adjustment for age, BMI, sex, smoking status, and family history of CRC. Furthermore, no evidence for effect modification of the association A986S variant and CRC by BMI, sex, or tumor site was observed. In addition, the A986S variant was not associated with the risk of obesity in controls and cases with CRC.

Table 2. The Genotype and Allele Frequencies of *Casr* Gene A986S (Rs1801725) Variant in Cases with Colorectal Cancer and Controls[a]

Variant	Controls (N = 354)	Cases (N = 303)	Crude		Adjusted[b]	
			OR (95% CI)	P Value	OR (95% CI)	P Value
rs1801725 G>T						
Genotype-wise comparison						
GG	215 (60.7)	181 (59.7)	1.0 (reference)		1.0 (reference)	
GT	121 (34.2)	107 (35.3)	1.05 (0.76-1.47)	0.768	1.09 (0.77-1.56)	0.619
TT	18 (5.1)	15 (5.0)	0.99 (0.49-2.02)	0.978	0.84 (0.38-1.83)	0.657
GT and TT	139 (39.3)	122 (40.3)	1.04 (0.76-1.43)	0.794	1.06 (0.76-1.48)	0.743
TT versus others	18 (5.1)	15 (5.0)	0.97 (0.48-1.96)	0.934	0.81 (0.37-1.76)	0.595
Allele-wise comparison						
G	551 (77.8)	469 (77.4)	1.0 (reference)		-	-
T	157 (22.2)	137 (22.6)	1.03 (0.79-1.33)	0.851	-	-

[a]Variables presented as No. (%).
[b]Adjusted for age, BMI, sex, smoking status, and family history.

Table 3. The Association Between CaSR Gene A986S (Rs1801725) Variant and Risk of Colon and Rectal Cancers After Adjustment for Age, BMI, Sex, Smoking Status, and Family History of Colorectal Cancer[a]

Variant	Control (N = 354)	Colon (N = 195)	OR (95%CI)	P Value	Control (N = 354)	Rectal (N = 108)	OR (95%CI)	P Value
rs1801725 G > T								
Genotype-wise comparison								
GG	215 (60.7)	113 (57.9)	1.0 (reference)		215 (60.7)	54 (50.0)	1.0 (reference)	
GT	121 (34.2)	74 (37.9)	1.16 (0.80-1.68)	0.420	121 (34.2)	43 (39.8)	1.11 (0.70-1.75)	0.654
TT	18 (5.1)	8 (4.1)	0.84 (0.35-2.00)	0.703	18 (5.1)	11 (10.2)	1.49 (0.69-3.22)	0.303
GT and TT	139 (39.3)	82 (42.0)	0.98 (0.69-1.39)	0.928	139 (39.3)	54 (50.0)	1.17 (0.76-1.80)	0.471
TT versus others	18 (5.1)	8 (4.1)	1.68 (0.93-3.04)	0.083	18 (5.1)	11 (10.2)	1.43 (0.68-3.00)	0.343
Allele-wise comparison								
G	551 (77.8)	278 (71.3)	1.0 (reference)		551 (77.8)	151 (69.9)	1.0 (reference)	
T	157 (22.2)	112 (28.7)	1.10 (0.84-1.45)	0.472	157 (22.2)	65 (30.1)	1.18 (0.84-1.65)	0.328

[a]Variables presented as No. (%).

4.1. CaSR Gene A986S Variant

Currently, CRC is considered as a complex disease that might result from the interaction between genetic and environmental factors. However, the number and nature of genes that influence susceptibility to CRC are largely unknown. The protective effect of calcium in CRC is well established and is the subject of recent interest. CaSR is an important component of the pathway through which calcium mediates its anticarcinogenic effects on the development of CRC. Calcium can prevent the development of colon cancer directly by inducing apoptosis and differentiation through binding to the CaSR, and indirectly by binding bile acids and free fatty acids (16). It is possible that the changed expression of CaSR is associated with abnormal differentiation and or tumor progression, or both (8). The CaSR expression patterns indicate its role in the pathogenesis of CRC; the expression of CaSR is high in normal large intestinal epithelium, is lower in well-differentiated colon cancer tissue, and is greatly decreased in undifferentiated carcinomas (17, 18). Furthermore, *CaSR* gene variants appear to be involved in maintaining calcium homeostasis (19). However, the influence of these variants on CaSR pro-

Table 4. The Association Between Genotypes and Alleles of *CaSR* Gene A986S (Rs1801725) Variant and Colorectal Cancer Risk According To Sex Category After Adjustment for Age, BMI, Smoking Status, and Family History of Colorectal Cancer[a]

Variant	Male				Female			
	Control (No. = 181)	Case (No. = 165)	OR (95%CI)	P Value	Control (No. = 173)	Case (No. = 138)	OR (95%CI)	P Value
rs1801725 G > T								
Genotype-wise comparison								
GG	99 (54.7)	88 (53.3)	1.0 (reference)		92 (53.2)	72 (52.2)	1.0 (reference)	
GT	72 (39.8)	60 (36.4)	1.09 (0.67-1.79)	0.707	65 (37.6)	49 (35.5)	1.06 (0.63-1.78)	0.819
TT	10 (5.5)	17 (10.3)	1.82 (0.73-4.53)	0.193	16 (9.2)	17 (12.3)	1.67 (0.76-3.70)	0.200
GT and TT	82 (45.3)	77 (46.7)	1.19 (0.75-1.90)	0.450	81 (46.8)	66 (47.8)	1.18 (0.73-1.90)	0.494
TT versus others	10 (5.5)	17 (10.3)	1.75 (0.72-4.25)	0.211	16 (9.2)	17 (12.3)	1.63 (0.76-3.50)	0.205
Allele-wise comparison								
G	270 (74.6)	236 (71.5)	1.0 (reference)		249 (72.0)	193 (69.9)	1.0 (reference)	
T	92 (25.4)	94 (28.5)	0.81 (0.59-1.11)	0.185	97 (28.0)	83 (30.1)	1.13 (0.81-1.57)	0.476

[a]Variables presented as No. (%).

Table 5. The Association Between *CaSR* Gene A986S (Rs1801725) Variant and Colorectal Cancer Risk According To BMI Category After Adjustment for Age, Sex, Smoking Status, and Family History of Colorectal Cancer[a]

Variant	Normal Weight (BMI < 25 Kg/m^2)				Overweight/Obese (BMI ≥ 25 Kg/m^2)			
	Control (No. = 180)	Case (No. = 146)	OR (95%CI)	P Value	Control (No. = 174)	Case (No. = 157)	OR (95%CI)	P Value
rs1801725 G > T								
Genotype-wise comparison								
GG	107 (59.4)	91 (62.3)	1.0 (reference)		108 (62.1)	90 (57.3)	1.0 (reference)	
GT	63 (35.0)	50 (34.2)	0.95 (0.57-1.58)	0.859	58 (33.3)	57 (36.3)	1.28 (0.78-2.10)	0.327
TT	10 (5.6)	5 (3.4)	0.52 (0.15-1.81)	0.311	8 (4.6)	10 (6.4)	1.14 (0.39-3.33)	0.805
GT and TT	73 (40.6)	55 (37.6)	0.89 (0.55-1.45)	0.658	66 (37.9)	67 (42.7)	1.26 (0.78-2.02)	0.355
TT versus others	10 (5.6)	5 (3.4)	0.53 (0.15-1.82)	0.319	8 (4.6)	10 (6.4)	1.04 (0.36-3.00)	0.931
Allele-wise comparison								
G	277 (75.9)	232 (79.5)	1.0 (reference)		274 (78.7)	237 (75.5)	1.0 (reference)	
T	88 (24.4)	60 (20.5)	0.81 (0.56-1.18)	0.278	74 (21.3)	77 (24.5)	1.20 (0.93-1.73)	0.319

[a]Variables presented as No. (%).

tein function is largely unknown up to now. Accordingly, these data support the hypothesis that the *CaSR* gene variants might have a role in pathogenesis of CRC.

To date, five epidemiological studies (10-14) have evaluated the association between the *CaSR* gene A986S variant and the risk of colorectal, colon, or rectal cancer. Previous studies have shown that *CaSR* is a very large gene and the rs1801725 G > T common variant, located in codon 986, resulting in an amino acid shift-alanine (A) or serine (S) - in the intracellular C-terminal tail of the CaSR. This variant (A986S) appears to be involved in maintaining calcium homeostasis and the "T" or "S" allele compared with the "G" or "A" allele was associated with higher circulating calcium and PTH concentrations (19, 20). However, the A986S

variant has little functionality despite being highly conserved (11). Studies of the effect of *CaSR* gene A986S variant on CRC have been inconclusive. Consistent with our findings, most previous studies found no association between this variant and CRC. We did not observe significant associations for the A986S variant in the overall analysis and in the analyses stratified by tumor site, sex, or BMI. In work by Speer et al. (10), the authors reported that there was no association between the A986S variant and rectal cancer risk in a population including 56 cases with rectal cancer and 112 controls. However, they found an association between this variant and more advanced rectal tumors. Another small study (11) that investigated the association between the A986S variant and CRC in 70 cases with CRC and 201 controls could not detect any association between the variant and CRC. On the other hand, Bacsi et al. (12) recently showed that the *CaSR* A986S "SS" genotype compared with "AA+AS" genotypes was more frequent in 278 cases with CRC than in 260 controls. However, in a large study of 1600 cases with colon cancer and 1949 controls which was conducted by Dong et al. (13) there was no significant association between the A986S variant and colon cancer overall. Their results suggested a possible role of some variants of the *CaSR* gene on proximal colon cancer, though. Finally, in another large study by Jenab et al. (14) including 1248 cases with CRC and 1248 controls, the A986S variant was not associated with CRC risk.

Inconsistent results such as these are unfortunately common in genetic association studies (21, 22) and discrepancy in these studies may be due to false positive results, differences in the genetic and/or environmental factors triggering the development of CRC, variation in dietary intakes including calcium, small sample size, and statistical methods. Alternatively, the A986S variant may be in linkage disequilibrium with another unknown functional variant of the *CaSR* gene that explains the discrepancy observed.

4.2. Study Limitations

Although well-designed, this study has several limitations. One limitation is the modest sample size that precludes drawing strong conclusions. Another limitation is that only one variant of the *CaSR* gene was genotyped and thus coverage of the gene was incomplete. The other limitation is colonoscopy-based study, and the population may not be representative of the general population. Accordingly, we could not completely rule out the possibility of chance findings. Nevertheless, the possibility of true finding should not be excluded.

In conclusion, in this case-control study, the A986S (rs1801725) variant located in exon 7 of *CaSR* gene not appear to affect the development of CRC in Iranian population. However, studies in other populations are warranted to confirm our findings.

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Footnotes

Authors' Contribution: Reza Dabiri: acquisition of data; administrative, technical, and material support; drafting of the manuscript. Touraj Mahmoudi: study concept and design; analysis and interpretation of data; drafting of the manuscript. Hamid Farahani: acquisition of data; statistical analysis; Hossein Nobakht: acquisition of data; drafting of the manuscript Mohammad Reza Zali: critical revision of the manuscript for important intellectual content; study supervision.

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