RESEARCH ARTICLE

Parathyroid Hormone Gene rs6256 and Calcium Sensing Receptor Gene rs1801725 Variants are not Associated with Susceptibility to Colorectal Cancer in Iran

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Abstract

Background: Substantial evidence from epidemiological studies has suggested that increased levels of calcium may play a protective role against colorectal cancer (CRC). Given the vital role of calcium sensing receptor (CaSR) and parathyroid hormone (PTH) in the maintenance of calcium homeostasis, we explored whether the rs1801725 (A986S) variant located in exon 7 of the CaSR gene and the rs6256 variant located in exon 3 of PTH gene might be associated with CRC risk. Materials and Methods: In this study 860 subjects including 350 cases with CRC and 510 controls were enrolled and genotyped using PCR-RFLP methods. Results: We observed no significant difference in genotype or allele frequencies between the cases with CRC and controls for both CaSR and PTH genes either before or after adjustment for confounding factors including age, BMI, sex, smoking status, and family history of CRC. Furthermore, no evidence for effect modification of any association of rs1801725 and rs6256 variants and CRC by BMI, sex, or tumor site was observed. In addition, there was no significant difference in genotype and allele frequencies between the normal weight (BMI <25 kg/m^2) cases and overweight/obese (BMI ≥25 kg/m^2) cases for the two SNPs. Conclusions: These data indicated that the CaSR gene A986S variant is not a genetic contributor to CRC risk in the Iranian population. Furthermore, our results suggest for the first time that PTH gene variant does not affect CRC risk. Nonetheless, further studies with larger sample size are needed to validate these findings.

Keywords: Colorectal cancer - CaSR - PTH - RFLP - gene variants - Iran

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Introduction

Colorectal cancer (CRC) is the second most commonly diagnosed cancer and the fourth leading cause of cancer-related mortality worldwide (Ferlay et al., 2010). Calcium appears to have anti-tumor activities and previous studies have indicated that dietary calcium may protect against the risk of colon cancer development or act as a confounding factor (Sesink et al., 2001; Galas et al., 2013; Morita et al., 2013). Calcium is involved in cell proliferation and differentiation and decreases colonic epithelial cell proliferation (Buset et al., 1986). Calcium and calcium sensing receptor (CaSR) are well-organized controllers of colonocytes and calcium by signaling through CaSR suppresses normal colonocyte proliferation (Whitfield, 2009). Furthermore, calcium stimulates the expression of the tumor suppressor gene E-cadherin via CaSR in colon cancer cells (Chakrabarty et al., 2003). Dysregulation of E-cadherin expression seems to be involved in progression from adenoma to carcinoma (Birchmeier, 1995). In addition, activation of CaSR suppresses β-catenin-TCF-4 oncogenic pathway (Chakrabarty et al., 2003; Varani, 2011).

CaSR which is expressed in human colonic epithelium and colon cancer cells (Hebert et al., 2004) contains seven exons and belongs to the G-protein coupled receptor superfamily. CaSR appears to play a tumor suppressor role in CRC and has a critical role in the maintenance of calcium homeostasis (Sarkar and Kumar, 2012; Ward et al., 2012) by modulating parathyroid hormone (PTH) secretion from the parathyroid glands (Saidak et al., 2009). In recent years, the relationship between CaSR gene polymorphisms and CRC risk has garnered a great deal of attention and association studies of CaSR gene rs1801725 (A986S) variant have been performed to investigate its implication with CRC risk, but despite the biological plausibility the results are inconsistent (Speer et al., 2002; Fuszek et al., 2004; Bacsi et al., 2008; Dong et al., 2008;

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Jenab et al., 2009) and the role of this gene in the etiology of CRC is still equivocal. Some studies (Speer et al., 2002; Fuszek et al., 2004; Dong et al., 2008; Jenab et al., 2009) have found no association, other investigations (Bacsi et al., 2008) have reported significant associations between the A986S variant and the risk of CRC.

PTH functions as another major regulator of calcium homeostasis and changes the expression of proteins involved in cell cycle in colon cancer cells (Calvo et al., 2011). Recent studies have shown increased serum levels of PTH in cases with CRC compared with controls (Charalampopoulos et al., 2010; Fedirko et al., 2011). Furthermore, previous studies have also demonstrated that PTH gene variants were associated with the serum levels of PTH and calcium (Kanzawa et al., 1999; Gohda et al., 2002). However, to our knowledge, no studies to date have evaluated the association between PTH gene variants and CRC risk.

Based on these considerations, we designed the present study to investigate the possible role of the rs1801725 variant located in exon 7 of CaSR gene and the rs6256 variant located in exon 3 of PTH gene in the etiology of CRC. These SNPs were chosen based on their degree of heterozygosity, position in the gene, functional importance, and use in previous genetic investigations.

Materials and Methods

Participants

A case-control study was performed with 510 controls (age range, 21-84 years) and 350 cases with CRC (age range, 20-89 years). All the subjects were Iranian and genetically unrelated. They were recruited between January 2009 and Jun 2012 by Gastroenterology and Liver Diseases Research Center, Shahid Beheshti University of Medical Sciences. Both cases with CRC and controls were recruited from individuals who were undergoing colonoscopy for various gastrointestinal (GI) complaints including long-term unexplained abdominal pain, unexplained weight loss, unexplained changes in bowel habit, rectal bleeding, chronic diarrhea, constipation, or screening program. However, the case group consisted of all eligible colonoscopy patients with positive pathologic report for CRC, while eligibility criteria for the control group included no individual history of malignant colorectal tumors, adenomatous polyps, or other polyps. Using self-administered questionnaire and before subjects' colonoscopy, information on demographic, anthropometric, and clinical characteristics of the cases and the controls was collected. Furthermore, laboratory personnel who performed genotyping were blinded to case or control status. Prior entering the study, informed consent was obtained from all study participants and the Ethical Review Boards of the Institution approved the study protocol. Study protocol was in accordance with the principles of the Helsinki Declaration. The body mass index (BMI) of each subject was calculated as weight (kg)/height (m^2) and the 810 subjects were divided in the subgroups based on the diagnosis of CRC and BMI values as follows: normal weight (BMI<25 kg/m^2) controls (n=251); overweight/obese (BMI≥25kg/m^2) controls (n=259); normal weight cases with CRC (n=169); and overweight/obese cases with CRC (n=181).

Genotype analysis

Genomic DNA was isolated from whole blood using standard methods, and genotyping was done by PCR-RFLP method. Characteristics of the studied gene variants and PCR and RFLP conditions are summarized in Table l. The PCR products were digested overnight by corresponding restriction enzymes (Fermentas, Leon-Rot, Germany) and then electrophoresed on 2 to 3% agarose gels. Bands in gels stained with ethidium bromide for visualization under ultraviolet light. CaSR and PTH genotypes of each subject were identified according to the digestion pattern and the presence or absence of the Hin1I and DraII sites, respectively. For quality control reasons, we repeated the genotyping analysis of approximately 10% of the randomly selected samples. The genotyping was also confirmed by the DNA sequencing of approximately 5% of the samples and all the results were concordant.

Statistical methods

Differences in demographic or anthropometric factors were calculated using t-test or chi-square (χ^2) test. The deviation of the genotype frequencies from Hardy-Weinberg equilibrium for each SNP among cases and controls was examined using χ^2 test. χ^2 test was also used to calculate the differences in the allele frequencies between the groups. Logistic regression was used to compare the distribution of the genotype frequencies between the different groups. To adjust confounding factors including age, BMI, sex, smoking statues, and family history of CRC logistic regression analysis was also used. The odds ratios (ORs) were the measure of associations and for all ORs, 95% confidence intervals (95% CI) were calculated. For statistical analyses, we used SPSS software, version 15.0 (SPSS Inc. Chicago, IL, USA) and a $p<0.05$ was considered statistically significant.

Results

Table 2 presents selected characteristics of the study cases and controls. There were no significant differences

Table 1. Information for the Studied Markers in CaSR and PTH Genes

Gene (SNP ID)	Location (Base change)	Forward Primer Reverse Primer	PCR program (35 cycles)	PCR fragment size (bp)	Resteriction enzym Incubation temperture	Alleles: RFLP fragments size (bp)
CaSR	Exon 7	5'-CTGAGCTTTGATGAGCCTCAGAAGGAC-3'	93ºC 45s,63ºC	269	"Hin1I, 37ºC	Allele T: 269
(rs1801725)	(G/T)	5'-CACTGATGACAAGCTCTGTGAACTGGA-3'	30s, 72 ºC 45s			Allele G: 241+28
PTH	Exon 3	5'-CATTCTGTGTACTATAGTTTG-3'	93ºC 45s,54ºC	600	"DraII, 37ºC	Allele A: 600
(rs6256)	(C/A)	5'-GAGCTTTGAATTAGCAGCATG-3'	30s, 72ºC 45s			Allele C: 420+180

between the cases with CRC and the controls according to their BMI, smoking status, and family history of CRC. However, the cases had significantly higher age compared with the controls (p<0.001). Furthermore, the percent of males was higher in cases with CRC than controls (p=0.015).

Table 3 shows the genotype and allele distributions for CaSR (rs1801725) and PTH (rs6256) gene variants in the cases with CRC and the controls. No significant deviation from Hardy-Weinberg equilibrium was found for each SNP in both cases and controls, suggesting that the alleles are in equilibrium (p>0.05). Furthermore, as shown in Table 3, we observed no significant difference in genotype or allele frequencies between the cases with CRC and controls for both CaSR and PTH genes either before or after adjustment for confounding factors including age, BMI, sex, smoking status, and family history of CRC. Additionally, when the subjects were stratified based BMI, sex, or tumor site (data not shown), again the frequency of CaSR and PTH genotypes and alleles were not significantly different between the groups either before or after adjustment for confounding factors.

Table 2. Study Population Characteristics[a]

Variables	Controls (n=510)	Cases (n=350)	P-value
Age (years)	44.0(16.3)	55.1(13.1)	<0.001
BMI(kg/m^2)	25.2(4.0)	25.2(4.4)	0.819
Gender			
Men	241(47.3)	195(55.7)	
Women	269(52.7)	155(44.3)	0.015
Smoking history			
No	433(84.9)	288(82.3)	
Former	61(12.0)	40(11.4)	
Current	16(3.1)	22(6.3)	0.087
Family history of colorectal cancer			
No	451(88.4)	311(88.9)	
Yes	59(11.6)	39(11.1)	0.832
Tumor site			
Colon	-	228(65.1)	
Rectal	-	122(34.9)	-

[a]Variables presented as mean (SD) or number (%)

Finally, risk of obesity in relation to the rs1801725 and rs6256 variants was also examined (data not shown). There were no significant differences between the normal weight controls and overweight/obese controls and between normal weight cases with CRC and overweight/obese cases with CRC with respect to distribution of the CaSR rs1801725 and PTH rs6256 genotypes and alleles, even if homozygous carriers of the minor allele and heterozygotes were combined.

Discussion

Although the mechanism of CRC remains unclear, both environmental and genetic factors are believed to work together and play an important role on the etiology of CRC (Kotnis et al., 2005). In complex multifactorial diseases such as CRC, it could be difficult to recognize a majority of genes because of their modest individual effects and complex interactions (Lusis et al., 2008). However, studying SNPs in candidate genes is an approach to identify novel susceptibility genes for the diseases. For this reason, investigation into the association between DNA sequence polymorphisms and CRC has become a subject of interest in recent years. Previous epidemiological studies have suggested that increased levels of calcium may play a protective role against CRC. With regard to the crucial role of CaSR and PTH in the maintenance of calcium homeostasis, our case-control study was design to clarify whether there is an association between CaSR and PTH gene polymorphisms and risk of CRC in Iranian population. The results of this study suggest that the rs1801725 variant of CaSR gene and the rs6256 variant of PTH gene are not associated with susceptibility to CRC either before or after adjustment for confounding factors. Moreover, no evidence for effect modification of the association the two calcium homeostasis-related genes and CRC risk by BMI, sex, or tumor site was found. In addition, there was no significant difference in genotype or allele frequencies between the CaSR and PTH gene variants and risk of obesity.

CaSR is an important component of the pathway

Table 3. Association between Genotypes and Alleles of CaSR and PTH Gene Variants and Colorectal Cancer Risk[a]

Gene (Variant)		Controls (n=510)	Cases (n=350)	OR (95% CI) P-value[b]
CaSR (rs1801725 G>T)				
Genotype-wise comparison	GG	302(59.2)	210(60.0)	1.0(reference)
	GT	178(34.9)	123(35.1)	1.00(0.73-1.36)0.999
	TT	30(5.9)	17(4.9)	0.76(0.39-1.49)0.425
	GT and TT	208(40.8)	140(40.0)	0.97(0.72-1.30)0.812
	TT versus others	30(5.9)	17(4.9)	1.31(0.68-2.54)0.418
Allele-wise comparison	G	782(76.7)	543(77.6)	1.0(reference)
	T	238(23.3)	157(22.4)	0.95(0.76-1.20)0.661
PTH (rs6256)[c]				
Genotype-wise comparison	CC	214(60.5)	189(62.4)	1.0(reference)
	CA	124(35.0)	96(31.7)	0.85(0.59-1.21)0.355
	AA	16(4.5)	18(5.9)	1.34(0.63-2.84)0.452
	CA and AA	140(39.5)	114(37.6)	0.90(0.64-1.26)0.543
	CC versus others	16(4.5)	18(5.9)	1.42(0.67-2.97)0.360
Allele-wise comparison	C	552(78.0)	474(78.2)	1.0(reference)
	A	156(22.0)	132(21.8)	0.99(0.76-1.28)0.912

[a]Variables presented as number (%); [b]Adjusted for age, BMI, sex, smoking status, and family history in genotype-wise comparisons; [c]Distribution of PTH gene variant in 354 controls and 303 cases

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 (Newmark et al., 1984). It is possible that the changed expression of CaSR is associated with abnormal differentiation and or tumor progression, or both (Chakrabarty et al., 2003). 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 (Gama et al., 1997; Sheinin et al., 2000). In fact, loss of the CaSR expression in colonic epithelium is a key event in the pathogenesis of colon cancer (Rogers et al., 2012). Furthermore, epigenetic inactivation of CaSR (CaSR methylation) has an important role in colorectal carcinogenesis (Hizaki et al., 2011). CaSR is a very large gene and its variants appear to be involved in maintaining calcium homeostasis (Cole et al., 1999). However, the influence of these variants on CaSR protein function is largely unknown up to now. The A986S 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. The A986S highly conserved variant 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 (Cole et al., 1999; Marz et al., 2007). Accordingly, these data support the hypothesis that the CaSR gene A986S variant might have a role in pathogenesis of CRC.

Studies of the effect of CaSR gene A986S variant on colorectal, colon, or rectal cancer have been inconclusive (Speer et al., 2002; Fuszek et al., 2004; Bacsi et al., 2008; Dong et al., 2008; Jenab et al., 2009). 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. (2002), 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 the variant and more advanced rectal tumors. Another small study (Fuszek et al., 2004) 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. In contrast, Bacsi et al. (2008) 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. (2008) 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. (2009) including 1248 cases with CRC and 1248 controls, the A986S variant was not associated with CRC risk. Unfortunately, inconsistent findings such as these are common in genetic association studies (Lohmueller et al., 2003). The discrepancy observed in the reported associations between CaSR gene variants and CRC risk may be explained by variations in the environmental, dietary or lifestyle factors triggering the development of CRC, ethnic or racial differences in genetic makeup, genotyped markers, small sample size, statistical methods, differences in disease definition, or false positive results. Furthermore, the discrepancy may be due to linkage disequilibrium between CaSR gene A986S variant and other unknown functional variants of CaSR gene.

The other gene studied here, PTH, is also involved in maintaining calcium homeostasis (Kanzawa et al., 1999). Interestingly, the present study was the first attempt to evaluate the association between PTH gene variant and CRC risk. We assessed the rs6256 polymorphism which is located in exon 3 of PTH gene and might contribute to the altered gene expression. It has been demonstrated that serum PTH levels were higher in subjects carrying the rs6256 "AA" genotype compared with individuals with the "AC" and "CC" genotypes (Kanzawa et al., 1999). Moreover, recent studies (Charalampopoulos et al., 2010; Fedirko et al., 2011) have reported that the serum level of PTH is higher in cases with CRC than controls. However, we did not find any significant association between PTH rs6256 genotypes or alleles and CRC risk in the overall analysis and in the analyses stratified by BMI, sex, or tumor site. It is possible that our sample size was not large enough to demonstrate the possible difference in the genotype or allele distributions between the different groups. However, to conclude that PTH gene is not involved in the pathogenesis of CRC, the rs6256 variant and other PTH gene variants should be investigated in other populations and larger studies.

Some potential limitations in the present study should be noted because they might have influenced our findings. First, is our relatively small sample size that may preclude drawing strong conclusions - in other words, our observations may be attributable strictly to chance. Second, is that by genotyping only one variant in CaSR and PTH genes, the coverage of the genes was incomplete. Third, we did not get the data of serum levels of PTH and calcium intake which could modify the effects observed here. Lastly, this study was hospital-based. Hence selection bias may exist and the population may not be representative of the general population. However, the genotype distributions of both control and case groups were compatible with the Hardy-Weinberg expectations, as well as the controls came from the same region with cases and were randomly sampled, which may reduce the effect of selection bias. In spite of these limitations, our study protocol was well designed and to our knowledge, this study is the first that examines the association between PTH gene variants and CRC risk and hence may serve to guide future studies in this area.

In summary, in this case-control study, the A986S variant located in exon 7 of CaSR gene not appear to affect the development of CRC in Iranian population. Furthermore, our results suggest for the first time that

PTH gene variant is not a genetic contributor to CRC risk. However, the results reported herein should be interpreted in the context of the study's limitations and thus further large-scale studies are required to confirm our observations.

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