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• *BASIC RESEARCH* •

Vitamin D receptor gene *Tru*9I polymorphism and risk for incidental sporadic colorectal adenomas

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Abstract

AIM: Recent laboratory and epidemiological studies suggest that vitamin D is a potential agent for colorectal cancer prevention. Its function is partially mediated by the vitamin D receptor (VDR). The aim of this study was to investigate whether a novel G (allele 'U')>A (allele 'u') polymorphism (*Tru*9I) in the VDR intron 8 region is associated with risk for colorectal adenoma in a colonoscopy-based case-control study.

METHODS: Genotyping for a total of 391 subjects was carried out through PCR and restriction fragment length polymorphism.

RESULTS: The frequencies of 'U' and 'u' alleles were 89.3% and 10.7%, respectively. The 'Uu' and 'uu' genotypes were associated with decreased risk for adenoma (OR, 0.71; 95%CI, 0.40-1.25). The inverse association was more pronounced for multiple adenomas and adenomas that were larger had moderate or greater dysplasia, or were sessile: the odds ratios (ORs) were, 0.51 (95%CI, 0.21-1.24), 0.37 (95%CI, 0.11-1.28), 0.68 (95%CI, 0.33-1.41), and 0.36 (95%CI, 0.13-0.97) respectively. In joint/combined analyses, inverse associations were more obvious among those who had at least one 'u' allele and also were younger (OR, 0.60; 95%CI, 0.26-1.37), women (OR, 0.38; 95%CI, 0.17-0.88), did not smoke (OR, 0.39; 95%CI, 0.13-1.23), or took NSAID (OR, 0.38; 95%CI, 0.12-1.25), but no evidence existed for interactions with calcium or vitamin D intake.

CONCLUSION: Our findings suggest that the VDR *Tru*9I polymorphism may be associated with lower risk for colorectal adenoma, particularly in interaction with various risk factors, but not with calcium or vitamin D.

Key words: Case-control study; Colorectal adenoma; Colorectal neoplasia; Vitamin D receptor; Genetic polymorphism

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers in the USA, with an annual incidence of 146 940 cases and 56 730 deaths[1]. It has been reported that in USA, CRC morbidity is greater in northern latitudes, which may in part be due to lower sun exposure[2]. Several epidemiological studies support the hypothesis that dermal vitamin D synthesis mediated by sunlight may protect against colorectal cancer; several found decreased risk for this disease with higher serum levels of vitamin D or with increasing dietary vitamin D intake[3-5]. The vitamin D receptor (VDR), a member of the steroid/thyroid receptor family, mediates genomic actions of the active metabolite of vitamin D [1, $25(OH)_2D_3$], and thus regulates cellular proliferation and differentiation[6-8] and induces apoptosis[9]. Recent studies show that VDR functions as a sensor of the colorectal carcinogen, lithocholic acid (LCA), inducing *in vivo* expression of the CYP3A family that detoxifies LCA in the liver and intestine[10]. Based on such findings, there has been increased interest in an interaction between vitamin D and the VDR gene and risk for colorectal cancer. Although contradictory results have been reported[11-13], evidence suggests that at least some VDR gene polymorphisms are related to the risk of CRC or adenoma[14-17].

Recently, a novel G>A polymorphism in the 3'-UTR region of the VDR gene was identified and designated as VDR *Tru*9I[18]. It is thought that the polymorphisms in this region of the VDR gene may affect its mRNA stability, possibly through linkage to other variants[19]. So far, no previous study has been reported on investigating the function of this polymorphism. In a epidemiological

study, Gyorffy *et al*.[20], found that the presence of the variant 'u' allele, combined with VDR *Apa*I 'a' and *Bsm*I 'b' alleles, is associated with increased risk for type I diabetes mellitus in girls. To our knowledge, there has been no previously published study on a potential association between the VDR *Tru*9I polymorphism and colorectal adenoma risk. Previously, we found that different genetic polymorphisms might affect risk for colorectal adenoma: the *cyclinD1 A/G* polymorphism was associated with increased risk[21], and the p53-inducible ribonucleotide reductase small subunit 2 (p53R2) 'AA' genotype was strongly associated with increased risk in those with lower dietary nutrients including vitamins and calcium intakes (paper submitted). Herein, we report data from this same North Carolina case-control study on the association of the VDR *Tru*9I polymorphism and colorectal adenoma risk, alone and in interaction with various environmental risk factors for colorectal neoplasms.

MATERIALS AND METHODS

Study design

From 1994 to 1997, the markers of adenomatous polyps (MAP) case-control study were conducted to assess the validity of colonic epithelial cell proliferation as a biomarker of risk for incident sporadic colorectal adenomatous polyps. Prior to beginning the study, MAP was approved by the Institutional Review Board of Wake Forest University, Bowman Gray School of Medicine in accordance with an assurance filed with and approved by the Department of Health and Human Services. Informed consent was obtained from each participant. Eligibility criteria for study subjects consisted of English speaking adults from 30 to 74 years of age, either sex or any race who were scheduled for elective outpatient colonoscopy by four large gastro-enterology practices in Winston-Salem and Charlotte, North Carolina. Patients were recruited for over 24 mo. Cases were identified as eligible colonoscopy patients who were determined to have study index pathologist-confirmed incident adenomatous polyps according to criteria adapted from the National Polyp Study[22]. Controls consisted of all eligible colonoscopy patients with no previous history of adenomatous polyps and who were found to be free of adenomatous polyps. Persons with familial polyposis, Gardner's syndrome, ulcerative colitis, Crohn's Disease, bowel resection, newly diagnosed recurrent adenomatous polyps, and incident colon cancer were excluded, as they were patients with past or prevalent cancer other than non-melanoma skin cancer.

Patients completed mailed questionnaires prior to their colonoscopies (to avoid bias) regarding family history of polyps or colon cancer, medical history, dietary information (via a semi-quantitative Willett 153-item Food Frequency Questionnaire), physical activity (via a modified Paffenbarger questionnaire), reproductive variables, body fat distribution, and their reasons for and the sequence of events leading to colonoscopy. Blood was drawn and stored at -70 ℃ for possible later measurement of various genotypes.

Preparation for colonoscopy included a 12 h fast and bowel cleansing with polyethylene glycol (GoLYTELY). Subjects willing to undergo biopsies had four quadrant biopsies taken from normal appearing mucosa in the rectum (10 cm above the anus), sigmoid colon, and cecum for a total of 16 biopsies. Information recorded included number of polyps, polyp size, polyp type (adenoma, hyperplastic, or mixed), adenoma subtype (tubular, villous, tubulo-villous), and the degree of dysplasia.

Subjects

Among all four clinical sites, 2 246 colonoscopy patients were identified. Of these, 669 were eligible on initial screening (eligibility rate 29.8%), and of these 633 were willing to discuss the study, 617 of these were contacted, and 417 of these signed consent and had study colonoscopies (consent rate 63.1%). Of the 417 participants, 259 had some type of polyp, and of these 179 had adenomatous polyps. Nine of the 417 patients were subsequently determined ineligible for the study, and an additional eight patients had incident colon cancer and were not eligible for the primary case-control analyses; thus, 400 possible patients were available for genotypic analysis. Of these 400 patients, viable DNA was isolated from 391 (171 cases and 220 controls) for genotyping.

Genotyping

Genomic DNA was obtained from stored WBCs digested in 500 μL of lysis buffer (50 mmol/L Tris-HCl, pH 8.5, 1 mmol/L EDTA, 0.2% SDS, 200 g/mL proteinase K) over night at 55 ℃ with shaking. The digestion was precipitated directly with isopropanol and the pellets were washed with 70% ethanol. The genomic DNA pellets (50-100 μg) were dissolved in 300-800 μL of TE buffer, of which about 1 μL was used for each PCR reaction. DNA was amplified following the primers designed for the exon 8 region of the VDR gene following the published DNA sequence (GenBank number: AY342401). An isoschizomer of *Tru*9I, *Mse*I, was used in this study. The PCR (50 μL volume) was carried out in 20 mmol/L Tris-HCl, pH 8.4, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTP, 0.5 mmol/L of each primer (5'-GCA GGG TAC AAA ACT TTG GAG -3' as forward and a 5'-CCT CAT CAC CGA CAT CAT GTC -3' as reverse), 80-120 ng of DNA template, and 2.5 units Taq polymerase (Gibco-Invitrogen). The solution was heated to 94 ℃ for 2 min, followed by 35 cycles of 30 s at 94 ℃, 30 s at 69 ℃, and 30 s at 72 ℃. The final reaction was extended 7 min at 72 ℃. The PCR products (5 μL) were loaded onto a 3% 2:1 NuSieve-SeaKem gel for confirmation. The PCR products (10 μL) were then subjected to *Mse*I restriction enzyme at 37 ℃ overnight. Bands for the wild-type ('UU') allele were not cut (177 bp); the 'uu' genotype was showed at 91 and 86 bp; and the heterozygote ('Uu') allele was cut into 177-, 91-, and 86-bp fragments.

Statistical analysis

Allelic frequencies for polymorphic VDR *Tru*9I G>A alleles were compared to those in previous study populations. VDR *Tru*9I G>A genotype (UU, Uu, uu) distributions for cases and controls were tested for adherence to the Hardy-Weinberg equilibrium.

All statistical inquiries were conducted using R language

version 1.9.0 from http://www.R-project.org. Descriptive comparisons (i.e., mean±SD, frequencies as percents) of cases and controls were conducted utilizing χ^2 tests for categorical variables, and *t* test for continuous variables.

Multiple logistic regressions were utilized to calculate odds ratios (ORs) and corresponding 95%CI, adjusted for potential concluding factors, to estimate the strength of an association between VDR *Tru*9I genotype and risk for incident sporadic colorectal adenomas. The effect of VDR *Tru*9I genotype was analyzed utilizing a priori hypothesized low risk, common 'UU' genotype as the referent group. A '*P*' test for trend was calculated across genotypes to detect a pattern of association.

Several risk factors were scrutinized as possible confounders or effect modifiers of the VDR *Tru*9I genotype-colorectal adenoma association. Among these were age, sex, race, body mass index, family history of colon cancer (FHCC), smoking, alcohol consumption, non-steroidal anti-inflammatory drug (NSAID) use, and total dietary intake of calcium and vitamin D. Criteria for inclusion of any covariate in the final model included: (1) biological plausibility; (2) whether it fits the model at $P \leqslant 0.1$; and (3) whether it altered the OR for the primary exposure variable by 10% or more. Final models for genotype main effects included age, sex, smoking status, drinking status, and FHCC. Models involving in the assessment of possible interactions between genotypes and various anti- and pro-proliferative and other key risk factors included age, sex, FHCC, NSAIDs, smoking status, and total intake of calcium and alcohol.

To examine potential gene-environment interactions of VDR *Tru*9I genotype and certain risk factors, stratified analyses were conducted. Continuous variables were dichotomized on median values for controls; furthermore, continuous dietary variables were categorized as sex-specific. Criteria for assessing effect modifiers were based on previous literature, biological plausibility, and whether or not risk estimates differed substantially across strata.

RESULTS

Adenoma cases were similar to the controls in respect to race, education status, and most dietary intakes (Table 1). However, cases were more likely to be a little older, male, and current drinkers or smokers. Controls were more likely to have histories of colon cancer in first-degree relatives.

There were significant differences in NSAID use and dietary calcium intake between cases and controls. The polymorphism distribution in present population was in Hardy-Weinberg equilibrium ($\chi^2 = 3.41$, $P = 0.07$).

Table 2 presents the associations between VDR *Tru*9I

Table 1 Selected characteristics of cases and controls, MAP study, 1994-1997[1]

	Cases (*n* = 171)	Controls (*n* = 220)	*P*[1]
Demographic factors			
Age (yr)[2]	58.4 (8.4)	55.8 (10.2)	0.006
Male (%)	60	36	<0.001
White (%)	89	89	0.98
College education (%)	19	23	0.38
Major risk factors			
Family history of colon cancer (%)	14	31	<0.001
Currently smoke cigarettes (%)	30	20	0.02
Currently drink alcohol (%)	75	55	<0.001
NSAID[3] use (%)	19	30	0.007
Dietary intakes			
Total energy (kcal/d)	2 010 (30)	2 169 (1 999)	0.27
Total fat (g/d)	71.3 (39.9)	72.6 (65.9)	0.80
Total meat (serve/wk)	4.4 (1.4)	4.5 (1.4)	0.57
Total fruits and vegetables (serve/wk)	6.1 (3.6)	7.4 (10.2)	0.09
Total calcium (mg/d)	736 (406.6)	871 (757)	0.02
Total vitamin D (IU/d)	315 (258.2)	359 (374)	0.16
Total folate (mg/d)	416.7 (241.6)	467 (402)	0.12
Total alcohol (g/d)	7.4 (15.1)	4.8 (20.8)	0.14

[1]Adjusted for age, sex, total energy intake, history of colon cancer in a first degree relative, nonsteroidal anti-inflammatory drug use, current smoking status, and total calcium and alcohol intakes; [2]mean±SD presented unless otherwise indicated, except for age, all other means are age adjusted; [3]non-steroidal anti-inflammatory drug.

Table 2 Frequencies of VDR *Tru9I* genotypes and associations with incident sporadic colorectal adenomas (MAP study), 1994-1997

VDR *Tru*9I genotype	Cases (*n* = 171)		Controls (*n* = 220)		Adjusted OR[1]	95%CI[2]
	n	%	*n*	%		
UU	144	84.2	171	77.8	1.00	
Uu	23	13.5	45	20.4	0.88	(0.17-4.55)[3]
uu	4	2.3	4	1.8	0.69	(0.38-1.25)
Uu+uu	27	15.8	49	22.2	0.71	(0.40-1.25)
P-trend					0.24	

[1]Odds ratio; [2]95% confidence interval; [3]adjusted for age, sex, total energy intake, history of colon cancer in a first degree relative, nonsteroidal anti-inflammatory drug use, current smoking status, and total calcium and alcohol intake.

genotypes and colorectal adenoma risk. The frequencies of VDR *Tru*9I 'UU', 'Uu', and 'uu' genotypes were 84.2%, 13.5%, and 2.3% in cases, and 77.8%, 20.3%, and 1.9% in controls, respectively. There were equivalent allele distributions for cases ('U' = 90.9%, 'u' = 9.1%) and controls ('U' = 88%, 'u' = 12%). A 29% decreased multivariable-adjusted OR (0.71; 95%CI, 0.40-1.25) was observed in 'Uu' and 'uu' carriers, compared to 'UU' carriers.

We investigated the association of the polymorphism with colorectal adenoma risk according to characteristics of adenomatous polyps (Table 3). The inverse association of having at least one 'u' allele with risk for colorectal adenoma was more pronounced for adenoma that were multiple (OR, 0.51; 95%CI, 0.21-1.24), larger (OR, 0.37; 95%CI, 0.11-1.28), sessile (OR, 0.36; 95%CI, 0.13-0.97), and perhaps for adenomas with higher levels of dysplasia (OR, 0.68; 95%CI, 0.33-1.41).

Potential interactions of VDR *Tru*9I polymorphism and other risk factors for colorectal neoplasms and risk for adenomas are shown in Table 4. Compared to the 'UU'

Table 3 Age-, sex-adjusted associations of VDR *Tru*9I genotypes and risk for incident sporadic colorectal adenomas according to adenoma characteristics, MAP Study, 1994-1997

Adenoma characteristics	*Tru*9I genotypes			
	UU		Uu+uu	
	Cases/controls (*n*)	OR[1]/95%CI[2]	Cases/controls (*n*)	OR/95%CI
Multiplicity				
1	85/171	1.00	20/49	0.88 (0.48-1.60)
>1	59/171	1.00	7/49	0.51 (0.21-1.24)
Shape				
Sessile	51/171	1.00	5/49	0.36 (0.13-0.97)
Pedunculated	97/171	1.00	22/49	0.93 (0.51-1.68)
Size (cm)[3]				
<1.0	111/171	1.00	24/49	0.84 (0.47-1.49)
≥1.0	33/171	1.00	3/49	0.37 (0.11-1.28)
Dysplasia[4]				
Mild	73/171	1.00	15/49	0.79 (0.41-1.53)
≥Moderate	71/171	1.00	12/49	0.68 (0.33-1.41)
Morphology[5]				
Tubular	131/171	1.00	27/49	0.80 (0.46-1.38)
Any villous	13/171	1.00	0/49	NA[6]

[1]Odds ratio; [2]95% confidence interval; [3]greatest diameter of largest adenoma; [4]dysplasia in adenoma with greatest degree of dysplasia; [5]if multiple adenomas, classified as "Any villous" if any adenoma villous or tubulovillous; [6]not available.

Table 4 Multivariate-adjusted[1] joint and combined associations of VDR *Tru9I* genotypes and various risk factors for colorectal neoplasms and risk for incident sporadic colorectal adenomas, MAP study, 1994-1997

	UU			Uu+uu		
	Cases (*n*)	Controls (*n*)	OR[2]/95%CI[3]	Cases (*n*)	Controls (*n*)	OR/95%CI
Age (yr)						
≤57	58	83	1.00	12	31	0.60 (0.26-1.37)
>57	86	89	1.41 (0.86-2.31)	15	18	1.09 (0.46-2.56)
Sex						
Male	85	60	1.00	15	16	0.65 (0.28-1.53)
Female	59	112	0.51 (0.30-0.85)	12	33	0.38 (0.17-0.88)
Current smoker						
No	35	77	1.00	6	25	0.39 (0.13-1.23)
Yes	105	94	1.84 (1.07-3.17)	20	24	1.56 (0.69-3.53)
Current drinker						
No	63	99	1.00	13	26	0.96 (0.44-2.08)
Yes	77	71	1.65 (1.01-2.69)	12	22	0.90 (0.39-2.06)
NSAID[4] use						
No	118	117	1.00	21	38	0.64 (0.34-1.23)
Yes	23	54	0.48 (0.26-0.86)	5	11	0.38 (0.12-1.25)
Total calcium intake[5]						
Lower	92	84	1.00	13	23	0.53 (0.23-1.20)
Higher	49	86	0.53 (0.32-0.87)	14	26	0.52 (0.23-1.14)
Total vitamin D intake[6]						
Lower	76	83	1.00	14	25	0.59 (0.26-1.33)
Higher	65	87	0.78 (0.48-1.27)	13	24	0.60 (0.27-1.38)

[1]Adjusted for age, sex, total energy intake, history of colon cancer in a first degree relative, nonsteroidal anti-inflammatory drug use, current smoking status, and total calcium and alcohol intakes; [2]odds ratio; [3]95% confidence interval; [4]nonsteroidal anti-inflammatory drug; [5]sex-specific median calcium intakes for males 701.3 mg/d, for females 754.1 mg/d; [6]sex-specific median vitamin D intakes for males 257.2 IU/d, for females 229.7 IU/d.

genotype carriers, having a 'u' allele was inversely associated with risk for colorectal adenoma among those who were younger (OR, 0.60; 95%CI, 0.26-1.37), women (OR, 0.38; 95%CI, 0.17-0.88), did not smoke (OR, 0.39; 95%CI, 0.13-1.23), or took NSAID (OR, 0.38; 95%CI, 0.12-1.25). Dietary calcium and vitamin D intake were observed to be related to decreased risk for adenoma; however, there was no evidence suggesting an interaction with the VDR *Tru*9I polymorphism.

DISCUSSION

VDR polymorphisms have been evaluated as risk factors for CRC or adenoma; however, their impact remains unclear. In the present study, we assessed, for the first time, VDR *Tru*9I variants as risk factor for colorectal adenoma. Our data suggest that the *Tru*9I mutant 'u' allele was associated with decreased risk for colorectal adenoma, particularly for adenomas that were larger, multiple, had moderate or greater dysplasia, or were sessile. Also, the 'u' allele was related to decreased risk for adenoma, particularly among persons who were younger, female, NSAID users or did not smoke.

Vitamin D is obtained from the diet or sunlight-induced synthesis, and hydroxylated first in the liver [forming 25-(OH)D], then subsequently in the kidney [forming 1,25-$(OH)_2$D]. The hypothesis that vitamin D may provide reduced colorectal adenoma risk was first proposed in the early 1980s in light of an inverse ecologic association between CRC morbidity and solar exposure[23]. *In vivo* and *in vitro* studies found that vitamin D_3 promotes differentiation of colon carcinoma cells by inducing E-cadherin and inhibiting β-catenin signaling[7]. Experimental data also suggest that the active metabolite of vitamin D and its analogs can induce apoptosis in a colorectal adenoma cell line[9]. Vitamin D interacts with the VDR, which upregulates CYP3A expression, which in turn increases detoxification of secondary bile acids, including LCA[10]. Recent epidemiological studies have suggested inverse associations among calcium, vitamin D and CRC or adenoma risk; but results are mixed. In the present study, higher calcium and vitamin D intake were associated with lower risk for colorectal adenomas; however, there was no support for the hypothesis that the VDR *Tru*9I polymorphism may interact with these dietary micronutrients.

VDR is thought to mediate most vitamin D effects, and six common polymorphisms of the VDR gene have been identified. It has been reported that the polymorphisms in the 3'UTR-region (*Bsm*I, *Taq*I, *Apa*I, *Tru*9I and *Poly-A*) might alter transcriptional activity and mRNA degradation[19]; and *Fok*I, located at the VDR start codon, affects the length of the N-terminal VDR transactivation domain, resulting in a three-amino acid longer protein[24]. Several epidemiological studies have addressed associations between VDR polymorphisms and different cancer types including colorectal cancers[11-15,25-31]. The *Fok*I polymorphism, which is a potential functional variant, has been extensively studied. Under various exposures, inconsistent results were obtained from different investigators: Wong *et al*.[14], reported, that the mutant 'f' allele was associated with increased risk of colorectal cancer; while another study by Ingles *et al*., found that the 'f' allele was related to decreased risk[13]; but null associations also have been observed[12,17]. The association of VDR *Bsm*I polymorphism with cancer risk has also been investigated. Two previous studies found that the VDR *Bsm*I 'BB' genotype may reduce risk for colorectal adenoma; in one, the association was modified by NSAID use, and in the other, the association was associated with reduced risk among those who had lower calcium intake and used NSAIDs[11,16]. Also, we previously reported that the VDR *Bsm*I variant 'b' allele was associated with colorectal adenoma risk in the same study population as the current study[32]. The VDR *Poly-A* polymorphism has been associated with decreased risk for colorectal adenoma, modified by NSAID use[11]. So far, there have been no previous reports of investigations of an association of the *Tru*9I polymorphism with any cancer.

Many studies have reported that the VDR polymorphisms are associated with susceptibility for and prognosis of different cancers[13,33,34]. Hutchinson *et al*.[33], found that the VDR 'ttff' genotype (according to *Taq*I and *Fok*I polymorphisms) was significantly associated with thicker malignant melanoma tumors. It has also been reported that the *Fok*I polymorphism was more strongly related to large adenoma risk among subjects with lower dietary calcium intake[13].

Some limitations in this study should be considered in interpreting our results. First, the small sample size and consequent low power preclude drawing strong conclusions. Second, this study is colonoscopy-based, and the population may not be representative of the general population. People who worried about their positive family history were more likely to seek colonoscopy examination, leading to a family history bias that may have attenuated associations. Another potential limitation is that ultraviolet radiation exposure was not assessed in the present study; therefore, the dietary vitamin D intake may not reflect the true exposure to vitamin D. In light of the relationship between vitamin D and calcium, this may also impact the estimated calcium-adenoma association.

In conclusion, this is the first study to investigate an association between the VDR *Tru*9I polymorphism and risk for incident sporadic colorectal adenoma. Our data suggest that the VDR *Tru*9I polymorphism may be more related to progression than initiation of colorectal adenoma. Our study also focused on the interaction between the VDR gene polymorphism, *Tru*9I, and dietary calcium and vitamin D intakes; however, no such interaction was found. On the other hand, our data suggest possible interactions of VDR *Tru*9I genotypes with age, sex, smoking, drinking, and NSAID use. Further, larger studies are needed to verify the present data, to understand the biological mechanisms of VDR gene/calcium/vitamin D interactions.

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