RESEARCH ARTICLE

Association of a VDR Gene Polymorphism with Risk of Colorectal Cancer in Kashmir

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Abstract

Roles of the vitamin D receptor in etiology of cancers, including colorectal cancer, have been repeatedly stressed in different parts of the world. A case control study aimed to evaluate the relationship between the two was therefore initiated in Kashmir, known both for its increasing incidence of gastrointestinal cancers and deficiency of micro-nutrients especially vitamin D. The study included a total of 617 subjects (312 colorectal cancer cases and 305 controls), with sampling carried out over a period of 5 years. DNA samples from the blood of the subjects were analyzed for start codon *Fok* I VDR polymorphism. We obtained a 1.3 fold increased risk among individuals homozygous for f variants as compared to subjects homozygous for F allele (odds ratio OR 1.3, 95%CI, 0.861-1.65). Our study also showed statistically significant results when dwelling and tumor location characteristics were stratified with *Fok* I polymorphism, all of which suggests a possible role of *Fok* I polymorphism in the etiology of CRC in Kashmir

Keywords: VDR *Fok* I polymorphism - colorectal cancer - cancer epidemiology

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Introduction

VDR is known to play an important role in a number of different pathways that include absorption of calcium in particular from intestines, metabolism of bones, immune cell differentiation and proliferation, as well as more importantly cellular processes of carcinogenesis, including differentiation, proliferation and apoptosis (Haussler et al., 1998; Lamprecht et al., 2001; Uitterlinden et al., 2004; Giovannucci et al., 2005; Ochs-Balcom et al., 2008). It is well known that Vitamin D mediates its action by binding to its cognate receptor and ultimately leads to the transcriptional activation or suppression of vitamin D responsive genes (Tajouri et al., 2005). Vitamin D the sunshine vitamin is known to play a protective role against the development of colorectal cancer as an inverse association has been observed between the serum concentration of vitamin D with colon cancer and colon adenomas. Polymorphisms in VDR genes have been studied recently in various parts of the world and the underlying variability has been suggested to influence the proliferation and differentiation of cells, and ultimately affect the downstream transcription of Vitamin D responsive genes (Ochs-Balcom et al., 2008). Till date more than 470 single nucleotide polymorphisms have been discovered on the human VDR gene, most of them are known to have low frequencies (McCullough et al., 2009). Particular stress has been laid on the fact that the VDR transactivation efficiency could potentially be influenced by a polymorphism in start codon as in *Fok* I region (Wong et al., 2003). It alters an ACG codon that is located ten base pairs upstream from the translation start codon and results in the generation of an additional start codon. If the initiating translation starts from this alternative site (thymine variant), it results in the generation of a longer VDR protein of 427 amino acids (Kostner et al., 2009). The polymorphisms in this area have been studied singly and in combination with other polymorphisms of VDR in a number of malignant and non malignant conditions (Chiu et al., 2001; Malecki et al., 2003; Park et al., 2006; Kadyska et al., 2007; Hubner et al., 2008; Neyestani et al., 2013).

Colorectal cancer is known in more general terms to be the cancer of epithelial origin that remains largely localized to the large intestine and rectum. It commonly occurs at some stage in approximately 5% of the population of the western world (Boyle et al., 2011). After metastasis has occurred, patient 5-year survival after surgery unfortunately falls dramatically from 90% to less than 10% (O'Connell et al., 2004). Kashmir has often being reported as a high-incidence area for gastrointestinal cancers (Murtaza et al., 2006). It has been found to be the

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fourth most common cancer in males, holds a third rank amongst the female population of Kashmir (Rasool et al., 2012) and has been observed to constitute 8.3% of all GIT cancers. An age-standardized incidence rate of 4.52 per 100,000 of population has been reported for this disease. Recent times have observed a remarkable progress in terms of the pace with which molecular studies including the polymorphic and mutational analysis have been carried on different GIT cancers in the valley that has given an insight about the predominance of variants present in our unique ethnic race (Hussain et al., 2011; Javid et al., 2011; Malik et al., 2011a; 2011b; 2011c; Rasool et al., 2011).

Materials and Methods

Study population

The sample collection for the study extended over a period of 5 years, until April 2013, subjects were recruited at Division of Gastroeneterology, Department of Medicine, Government Medical College, Srinagar, Kashmir. The recruitment process was initiated following the approval from the ethical committee of Government Medical College, Srinagar. The diagnosis of CRC was based on the standard colonoscopic/sigmoidoscopic methods (flexible type) and histopathological criteria. Controls were taken from healthy individuals of Kashmir valley from same division, of Government Medical College, Srinagar following the referral pattern of sex and age matched patients. None of the controls had a personal history of malignancy. At recruitment, informed consent was obtained from each subject and personal data from each participant regarding demographic characteristics, such as sex, age, and related risk factors including smoking were collected via questionnaire. A 3-5ml of venous blood sample was obtained from each subject and transferred into an EDTA coated vial. This was done after cleaning the area with 100% alcohol swab and using 5ml dispovan syringe. The collected samples were then immediately shifted and stored at -80°C until DNA was extracted. A total of 657 samples were collected from subjects, 17 of them did not cooperate or were ready for the consent, 12 provided incomplete information and 8 samples did not yield desired amount of DNA, leaving a total of 617 samples useful for the present study. Present study thus included a total of 312 colonoscopically and histologically confirmed CRC patients called as cases and 305 controls, 23 of the cases had a family history of cancer. Commercially available, genomic DNA purification kit (Genetix Biotech Asia, Pvt Ltd.) was used to extract the DNA from the blood samples of Subjects both cases and controls.

Genotyping

Desired region of the genome was amplified using an oligonucleotide primer set consisting of *Fok* I- for, 5-AGC TGG CCC TGG CAC TGA CTC TGC TCT-3 and *Fok* I- rev, 5-ATG GAA ACA CCT TGC TTC TTC TCC CTC-3 sequences, that resulted in the amplification of 265bp fragment. PCR was carried out in a final volume of 25µl reaction mixture that consisted of 50-75 ng of genomic DNA template, 0.5µM of each primer (Genescript), 12.5µl of Maxima Hot start master mix (Fermentas/ Thermoscientific) (along with nuclease free water added accordingly). The conditions were selected after extensively standardizing all the parameters. PCR technique applied to amplify the polymorphic site of *Fok* I, consisted of an initial denaturation for 5 min, followed by 35 cycles each of 30s at 94°C, 30s annealing phase at 62°C and 30s extension phase at 72°C and ultimately a 7 min final extension at 72°C was used to complete the reaction. As the gain of restriction site occurs in the polymorphic allele, amplicons were treated with *Fok* I (Fermentas) (1U at 55°C) enzyme and kept for 4 hours that resulted in the restriction of respective variants according to the number and presence of restriction sites. Resulting genotypes were denoted accordingly as *FF* (265) *Ff* (265, 196 and 69) or *ff* (196 and 69) (*FF* was used to denote complete absence of restriction site in any of the alleles) and electrophoresed on ethidium bromide treated 2.5% agarose gel (Sigma Aldrich).

Statistical analysis

GraphPad Prism version 5.0, software was used to carry out the statistical analysis on the data. χ^2 test was used to examine difference in terms of genotypic distribution and to examine differences among demographic variables. Allelic frequencies were determined by gene counting. Association between genotype and risk (genotypic risk magnitudes or effect size) were estimated by calculating odds ratio (OR) with 95% confidence intervals (95%CIs).

Results

Present study included a total of 172 male and 140 female cases (M/F-1.23), and 155 male and 150 (M/F-1.03) female control subjects. Table I presents selective general characteristics of cases and controls that were included in the present study. Mean age calculated for cases was 52.05 years and that of controls was 51.06 years.

Since no significant differences were observed between cases and controls with respect to various characteristics ($p>0.05$), it suggested that frequency matching was adequate.

Table 2 shows the allele and genotypic frequencies of different polymorphic variants among cases and controls, resulting from the SNP in exon 2 of VDR. While as the genotypic and allele distribution of the *Fok* I polymorphism among cases is given in Table 3.

Out of 312 cases, 88(28.2%) belonged to urban region,

Table 1. General Characteristics of Study Population (cases and controls)

Characteristics		Cases (312)	Controls (305)	p value*
Age	≤50	148	150	0.66
	>50	164	155	
Gender	Male	172	155	0.28
	Female	140	150	
Dwelling	Urban	88	90	0.71
	Rural	224	215	
Smoking/Snuff	Ever	152	140	0.48
	Never	160	165	

224 (71.79%) to rural ones; 148 (47.43) were either below or of 50 years of age, 164 (52.56%) where above 50 years of age and 156 (50%) carried out any type of smoking (Hukka or Cigarette either singly or in combination) or used to snuff. 164 (52.56%) patients carried the malignancy in colonic region and 148 (47.43%) had it in rectal. 56.41% patients were found to be homozygous for the occurrence of *FF* genotype, 34.61% were heterozygous for the SNP and only 8.9% had the risk allele, where as the frequency of alleles among controls was found to be 54.09%, 39.34% and 6.55% implying frequency to be more in colorectal cancer patients than in controls. *ff* genotype was relatively more frequent (71.42%) among male cancer patients or those above 50 years of age or who had cancer in the colon. On contrary *FF* and *Ff* genotype (F allele) was most predominant among cases belonging to rural areas.

To evaluate the significance of *Fok* I polymorphism individually, we further stratified it in colon cancer patients Table 4 shows association of *Fok* I variants among the different categories of colon cancer patients.

Study showed that there was an equal distribution of smokers and non smokers among general colorectal cancer and colon cancer groups. Majority of the colon cancer patients came from rural areas. As some categories showed complete absence of *ff* genotype, when age of patients (cases) was further sub categorized, two general categories (≤50 and >50) were included in the initial analysis to begin with. Figure 1, shows the occurrence of *Fok* I genotype among different age groups. The figure clearly indicates more occurrence of *ff* genotype in persons belonging to age group, 51-60. The 41-50 age group category, showed a predominance of *FF* and *Ff* genotypes (F allele).

Although our study did not show an overall statistically significant association of *Fok* I (T/C), VDR polymorphism with the risk of CRC, we found a 1.3 fold increased risk of colorectal cancer (95%CI, 0.861-1.65) among individuals with *ff* genotype (risk allele) when compared against *FF* genotype possessing individuals, suggesting subjects homozygous for "f" allele, might be at risk than individuals carrying homozygous "F" allele. Our study also showed statistically significant results when dwelling and tumor location characteristics were stratified with *Fok* I polymorphism. Study on contrary showed no significant association between *Fok* I polymorphism and age or smoking status among colorectal cancer patients in general or with dwelling in rectal cancer patients or with smoking status in both individual colon and rectal cancer patient categories. Which means our study showed no association between smoking and *Fok* I polymorphism in cancer patients of Kashmir, irrespective of tumor location. We found a significant association of *Fok* I polymorphism in males with either colorectal cancer or cancer in colon only.

Table 2. Genotypic and Allelic Frequencies of *Fok* I, VDR, among Case and Controls and Their Association with Risk of CRC

Gene	Variants	Cases (312)	Controls (305)	O.R (95%CI)		p value
Fok I	*FF*	176 (56.41%)	165 (54.09%)	1.00		
	Ff	108 (34.61%)	120 (39.34%)	0.84	(0.60-1.18)	0.32
	Ff	28 (8.9%)	20 (6.55%)	1.3	(0.861-1.65)	0.38
	Ff+ff	136 (77.27)	140 (84.84)	0.917	(0.663-1.251)	0.56

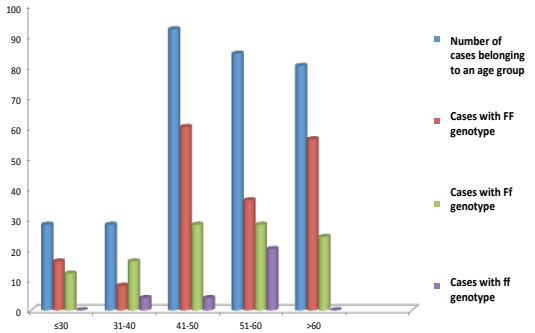

Figure 1. Distribution of *Fok* I Genotypes among Different Age Groups

Table 3. Clinicopathological Characteristics of Colorectal Cancer Patients and *Fok* I DNA Polymorphism

Variables		Total	*FF* n=312	*Ff* 176	*ff* 108	χ^2, p value 28
Age	≤50	148 (47.43)	84 (47.72)	56 (51.850)	8 (28.57)	4.85, 0.08
	>50	164 (52.56)	92 (52.27)	52 (48.14)	20 (71.42)	
Gender	Male	172 (55.12)	104 (59.09)	48 (44.44)	20 (71.42)	**9.11, 0.01**
	Female	140 (44.87)	72 (40.9)	60 (55.55)	8 (28.57)	
Dwelling	Urban	88 (28.2)	40 (22.72)	32 (29.62)	16 (57.14)	**14.3, 0.0008**
	Rural	224 (71.79)	136 (77.27)	76 (70.37)	12 (42.85)	
Smoking/Snuff	Ever	156 (48.71)	88 (50)	52 (48.14)	16(57.14)	0.72, 0.697
	Never	156 (51.28)	88 (50)	56 (51.85)	12 (42.85)	
Location of cancer	Colon	164 (52.56)	100 (56.81)	48 (44.44)	20 (71.42)	**7.95, 0.018**
	Rectum	148 (47.43)	76 (43.18)	60 (55.55)	8 (28.57)	

Table 4. Significance of *Fok* I Polymorphism with Respect to Characteristics of Colon Cancer Patients

Variables		Total n-168	*FF* 100	*Ff* 48	*ff* 20	χ^2, p value 28
Dwelling	Urban	40 (23.8)	20 (20)	8 (16.66)	12 (60)	8.27,0.016
	Rural	128 (76.19)	80 (80)	40 (83.33)	8 (40)	
Gender	Males	80 (47.61)	48 (48)	16 (33.33)	16 (80)	12.34, 0.0021
	Females	88 (52.38)	52 (52)	32 (66.66)	4 (20)	
Smoking status	Smoker	84 (50)	44 (44)	28 (58.33)	12 (60)	3.57,0.16
	Nonsmoker	84 (50)	56 (56)	20 (41.66)	8 (40)	

Discussion

Although some attempts have already been made to evaluate the role of *Fok* I (C/T), VDR polymorphism in various types of cancer, including colorectal cancer (Correa-Cerro et al., 1999; Curran et al., 1999; Ingles 2000; Bretherton-Watt 2001; Chokkalingam 2001; Wong et al., 2003). No such studies directed to unravel the role of Vitamin D and its receptor gene, have been carried out so far in Kashmir valley, which represents the Northern most part of India (Rasool et al., 2012). People in this corner of World are very much prone to deficiencies (especially to Vit D deficiency) because of their distinct and unique dietary habits and culture.

Taking these factors into account, we assessed *Fok* I SNP of VDR in our population for the first time, to our knowledge, and found persons with *ff* homozygous genotype at a risk than individuals having *FF* genotype. *Fok* I polymorphism is known to alter ATG start codon to ACG, that shortens the resulting receptor protein by three amino acid length (Arai et al., 1997; Miyamato et al., 1997; Gross et al., 1998; Jurutka et al., 2000) which is represented by F. This F allele has been repeatedly suggested to transmit stronger anti-proliferative and pro-differentation signals, by interacting with TFIIB (Jurutka et al., 2000; Whitfield et al., 2001; Wong et al., 2003). This observation in our ethnic population is consistent with some of the previous observations found among different races in different parts of the world (Arai et al., 1997; Jurutka et al., 2000).

The association of *Fok* I polymorphism with males in case of colorectal cancer and in colon cancer alone points towards complex interactions between gender-related differences in exposure to hormones and risk factors, and how they interact with two different kinds of VDR proteins, something observed previously (Ochs-Balcom et al., 2008). Our study also yielded a positive statistical interaction between the occurrence of colon cancer and *Fok* I polymorphism, which was consistent with the studies carried out by Balcom et al. (2008) and Wong et al. (2003).

Quite surprisingly a strong association was observed when *Fok* I polymorphism was stratified with dwelling (subjects with a frequency distribution of 77.27% and homozygous for F allele and subjects with a frequency of 70.37% and heterozygous for F genotype belonged to rural areas); and *Fok* I polymorphism was stratified with rural colon cancer subjects (*FF*, 80% and *Ff*, 83.3%) possible reasons for such an observation may be, deficiency of micronutrients like folate, Vitamin B6, Vitamin B12 and methionine that have been reported to protect against cancer by preventing against abberrant DNA methylation patterns that are commonly seen in colorectal tumors (Feinberg et al., 1987; Kovacic et al., 2001; Borek et al., 2004; Timbo et al., 2006). Deficiency of these micronutrients remains high among under-privileged rural class, moreover they are relatively high consumers of tobacco (in the form of Hukka), beef, sun dried vegetables and salted tea (both very rich source of carcinogens), all of these factors predominating among the rural back ward classes of Kashmir, have been suggested to have a strong role to play in the etiology of cancers (Chakravarti et al., 1975; Siddiqi et al., 1988; Dar et al., 2012; Rasool et al., 2012). Providing evidence to suggest that their cumulative effect may greatly mask the protective effect of "F" allele among Kashmiri rural dwellers. So this observation supports the notion that genetic effects can be easily overwhelmed by the environmental effect.

In conclusion, our study demonstrated importantly a 1.3 fold increased risk of CRC among individuals with homozygous *ff* genotype than with *FF* genotype. The study indicated a significant association of *Fok* I polymorphism among males in general and those having cancer in the colon area. However to evaluate further the impact of this polymorphism on the development and prognosis of CRC, more exhaustive studies on a large scale need to be carried out on our population.

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