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

Sabha Rasool1, Showkat Ahmad Kadla2, Tanzeela Khan1, Falak Qazi1, Nisar Ahmad Shah2, Javed Basu2, Bilal Ahmad Khan2, Qulsum Ahktar1, Aga Syed Sameer3, Bashir Ahmad Ganai1*

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 Gastroenterology, 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 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, (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, 169 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. χ² 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.

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 homzygous 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 homzygous for “f” allele, might be at risk than individuals carrying homzygous “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) |
|---------------|------------|----------------|-------------|
| 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 (44.87) | 140 (44.84) | 0.917 (0.663-1.251) | 0.56 |

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

| Variables      | Total | FF (m=312) | Ff | ff | χ², p value |
|----------------|-------|------------|----|----|-------------|
| Age ≤50        | 148 (47.43) | 84 (47.72) | 56 (51.850) | 8 (28.57) | 4.85, 0.08 |
| Age >50        | 164 (52.56) | 92 (52.27) | 52 (48.14) | 20 (71.42) | 9.11, 0.01 |
| Gender Male    | 172 (55.12) | 104 (59.09) | 48 (44.44) | 20 (71.42) | 14.3, 0.0008 |
| Female         | 140 (44.87) | 72 (40.9) | 60 (55.55) | 8 (28.57) | 0.72, 0.697 |
| Dwelling Urban | 88 (28.2) | 40 (22.72) | 32 (29.62) | 16 (57.14) | 7.95, 0.018 |
| Rural          | 224 (71.79) | 136 (77.27) | 76 (70.37) | 12 (42.85) | 7.95, 0.018 |
| Smoking/Snuff |          |            |    |    |             |
| Ever           | 156 (48.71) | 88 (50) | 52 (48.14) | 16 (57.14) | 7.95, 0.018 |
| Never          | 156 (51.28) | 88 (50) | 56 (51.85) | 12 (42.85) | 7.95, 0.018 |
| Location cancer| Colorectal | 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) | 7.95, 0.018 |

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

| Variables      | Total n=168 | FF (m=100) | Ff | ff | χ², p value |
|----------------|-------------|------------|----|----|-------------|
| 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) | 12.34, 0.0021 |
| Gender Males   | 80 (47.61) | 48 (48) | 16 (33.33) | 16 (60) | 3.57, 0.16 |
| Females        | 88 (52.38) | 52 (52) | 32 (66.66) | 4 (20) | 12.34, 0.0021 |
| 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) | 3.57, 0.16 |
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; Miyamoto 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-differentiation signals, by interacting with TFII B (Jurutka et al., 2000; Whittlefield 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 be, deficiency of micronutrients like folate, Vitamin B6, 83.3%) possible reasons for such an observation may.

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