Original article

Association of Vitamin D Receptor Polymorphisms (*FokI* (Rs2228570), *ApaI* (Rs7975232), *BsmI* (Rs1544410), and *TaqI* (Rs731236)) with Gastric Cancer in a Kurdish Population from West of Iran

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Abstract

Background: The association of 1,25-dihydroxy vitamin D3 (1,25(OH)2D3) and its receptor, vitamin D receptor (VDR), with cancer types have been studied. However, there are controversial findings regarding the association of specific VDR polymorphisms with different kinds of cancers. In the current study, we investigated the association of VDR polymorphisms (Fok1 (rs2228570), *ApaI* (rs7975232), *BsmI* (rs1544410), and *TaqI* (rs731236)) with the risk of gastric cancer in a Kurdish population of Kermanshah in Iran for the first time.

Methods: In this case-control study, the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used in 99 gastric cancer patients and 100 healthy subjects as controls.

Results: The frequencies of f (*FokI*), b (*BsmI*), t (*TaqI*), and a (*ApaI*) alleles were: 55.6%, 27.3%, 62.1%, and 44.95% in the patient group, respectively and 42%, 29.5%, 54.5%, and 46.0% in the control group, respectively. Analysis of the results indicated that there was a positive association between the frequency of *FokI* genotypes with gastric cancer risk ($p= 0.021$). However, no statistically significant association of *BsmI*, Taq1, and *ApaI* polymorphisms of VDR was detected in gastric patients when compared with healthy individuals.

Conclusions: VDR-*FokI* polymorphism could increase the risk of GC development and predispose to the disease by mechanisms.

Keywords: Gastric cancer, PCR-RFLP, Polymorphism, Vitamin D receptor.

Introduction

Gastric cancer, as the second most prevalent tumor worldwide, originates from the lining of the stomach and is a significant global threat to public health (1-3). Gastric carcinogenesis is a multifactorial process in which genetic and environmental factors are both implicated (4). In this regard, the vitamin D receptor (*VDR*) gene has been a research focus due to its essential roles in the regulation of cell proliferation, differentiation as well as apoptosis. The *VDR* maps to the chromosome region 12q13.1 with a length of 63493 nucleotides and spans approximately 75 kb (5, 6).

The *VDR*, as a member of the steroid hormone receptor family, specifically binds vitamin D or 1,25(OH)$_2$D mediating its effects

(7, 8). As one of its key roles, vitamin D can affect cancer growth and expansion via multiple signaling pathways implicated in cell apoptosis, proliferation, invasion, and metastasis. Low $1,25 (OH)_2 D_3$ serum levels are associated with cancer progression (9). Various studies by elucidating their anticancer activities and the related underlying mechanisms have addressed the involvement of VDR and vitamin D in cancer. In 1981, $1,25(OH)_2D_3$ showed its ability to significantly inhibit the proliferation of melanoma cells *in vitro* at Nano molar concentrations (10). Various VDR signaling-mediated mechanisms account for the mentioned anti-tumor effects. For instance, VDR signaling can induce G0/G1 cell cycle arrest coupled with upregulation of several inhibitors of cell cycle like P21 and P27. Also, it can induce cell cycle arrest at G2/M and increase the expression of E-cadherin and desmosomes that lead to apoptosis and enhance adhesion and suppress the migration of cancer cells. Epidemiological evidence has highlighted an inverse correlation between the intensity of local sunlight and the risk of some cancers, allegorically prostate and breast cancer (9). Also, VDR-Knock out mice shows a higher tendency to develop cancer following challenge with carcinogens (11). The *VDR* gene variants, including *BsmI* (rs1544410, intron 8, $+63980\,G > A$), *TaqI* (rs731236, exon 9, $+65058\,T > C$), and *ApaI* (rs7975232, intron 8, $+64978\,C > A$) have been located in the 3' end region of the VDR gene, whereas *FokI* (rs2228570, rs10735810, exon 2, $+30920\,C > T$) is found at the translation start codon (12, 13). Previous studies have revealed that single nucleotide polymorphisms (SNPs) of the *VDR* gene (*BsmI*, *TaqI*, *ApaI*, *FokI*) associate with the risk of cancer development (14-16).

The current study aimed to study the association and role of the *VDR* gene polymorphisms in gastric cancer and the frequencies of VDR-Fok1, VDR-*ApaI*, VDR-*BsmI*, and VDR-*TaqI* polymorphisms in patients with gastric cancer compared to healthy individuals.

Materials and Methods

Materials
The DNA extraction kit, agarose, Taq polymerase, MgCl2, buffer, and dNTPs used for polymerase chain reactions (PCR) were purchased from Pishgam Co. (Tehran, Iran). The *BsmI*, *ApaI*, *TaqI*, and *FokI* restriction enzymes were from Thermo Scientific Fermentas. All other reagents and chemicals were obtained from Sigma (USA). The primers were synthesized by Pishgam Co. (Tehran, Iran).

Sample collection
A total of 99 patients (47 males and 52 females) with gastric cancer with a mean age of 56±8.43 years and 100 healthy controls (56 males and 44 females) with a mean age of 37±7.43 years were enrolled in this study. Five mL of the blood samples were obtained from every patient and control individuals. All the studied individuals had the Kurdish ethnicity and were from the west of Iran. Ethics Committee in Kermanshah University of Medical Sciences, Iran, approved the current study (No. 97402). All individuals agreed and signed the form of informed consent (Helsinki II declaration).

DNA extraction and Genotyping
The extraction of genomic DNA from peripheral blood samples was carried out using the DNA extraction kit (Pishgam Co.). To investigate VDR-*ApaI*, *BsmI*, *FokI*, and *TaqI* polymorphisms, the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique was applied (17, 18). Specific PCR primers were designed using robust oligo primer design software and SNP database (dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP) (Table 1).

Statistical Analysis
Immunoblot data were quantified by densitometry and presented in means±standard deviation of at least two replicates. Statistical analysis was accomplished using Student's t-test in Microsoft Excel, with p-values less than 0.05 considered as statistically significant.

Table 1. The sequence of the primers and PCR–RFLP products characteristics.

Primer name	Sequence	PCR product (bp)	Digested length (bp)
FokI-F	TCCCTGGCACTGACTCTGGCTTT	259	194, 65
FokI-R	GAAACACCTTGCTTCTTCTCCGT		
BsmI-F	CAACCAAGACTACAAGTACCGC	822	648, 174
BsmI-R	AACCAGCGGAAGAGGTCAAGG		
TaqI-F	AGAGCATGGACAGGGAGCAAG	744	247, 497
TaqI-R	GCAACTCCTCATGGCTGAGGTCT		
ApaI-F	AGAGCATGGACAGGGAGCAAG	744	212, 532
ApaI-R	GCAACTCCTCATGGCTGAGGTCT		

The used primers are presented in Table 1. The PCR-RFLP method was performed in 25 μL reaction volumes containing 10X buffer (100 mM Tris-HCl, 50 mM $MgCl_2$, 500 mM KCl, pH 8.3), 0.2 mM dNTPs, 200 ng template DNA, and 1 U AmpliTaq DNA polymerase. The touchdown PCR program was performed. The following steps, for *FokI*: one cycle of initial denaturation for 10 min at 95 °C, then 35 cycles of denaturation for 45 sec at 94 °C followed by annealing for 45 sec at 62 °C, and final extension for 10 min at 72 °C; for *BsmI*: one cycle of initial denaturation for 10 min at 95 °C, then 35 cycles of denaturation for 45 sec at 94 °C followed by annealing for 45 sec at 60 °C, and final extension for 7 min at 72 °C; for *TaqI*: one cycle of initial denaturation for 10 min at 95 °C, then 35 cycles of denaturation for 45 sec at 94 °C followed by annealing for 45 sec at 61 °C, and final extension for 10 min at 72 °C; and for *ApaI*: one cycle of initial denaturation for 10 min at 95 °C, then 35 cycles of denaturation for 45 sec at 94 °C followed by annealing for 40 sec at 58 °C and final extension for 10 min at 72 °C.

The PCR products were separately digested for 24 h with *FokI*, *BsmI*, *TaqI*, and *ApaI* restriction enzymes at 37 °C overnight for *FokI*, *BsmI*, *TaqI*, and *ApaI* SNPs, respectively (19). The digested PCR products underwent electrophoresed on 2% agarose gels and were detected via staining with GelRed DNA stain. For genotype sequencing, the samples were analyzed by a DNA sequencer apparatus (Macrogen, South Korea).

Statistical analysis
The genotype frequencies of VDR polymorphisms were compared using the Pearson's Chi-squared test in the IBM SPSS software using statistical package for social sciences (SPSS) version 18 and logistic regression analysis with 95% confidence intervals (CIs).

Results
Table 2 shows the frequency distribution of alleles and genotypes for the patients and control groups. Anal oysisf the 199 subjects, 99 gastric cancer patients and 100 healthy controls, showed that the frequency oft and f alleles was higher in the gastric cancer cases (t= 62.1%, f= 55.6%) compared to controls (t= 54.5%, f= 42%) while the frequencies of a and b alleles were lower in the gastric cancer cases (a= 44.95%, b= 27.3%) compared to controls (a= 46%, b= 29.5%) as presented in Table 2.

The frequencies of f (*FokI*), b (*BsmI*), t (*TaqI*) and a (*ApaI*) alleles were: 55.6%, 27.3%, 62.1% and 44.95% in the patient group, respectively and 42%, 29.5%, 54.5%, and 46.0% in the control group, respectively. Analysis of the results indicated that there was a positive association between the frequency of *FokI* genotypes with gastric cancer risk (p= 0.021). However, no statistically significant association of *BsmI*, Taq1, and *ApaI* polymorphisms of *VDR* was detected in gastric patients when compared with healthy individuals. The results of direct DNA sequencing of the patients with wild

genotype and mutant genotype for each polymorphism are also shown in Figure 1.

Table 2. Genotype and allele frequency (in percent) of *BsmI*, *FokI*, *TaqI*, and *ApaI* in gastric cancer cases and healthy controls.

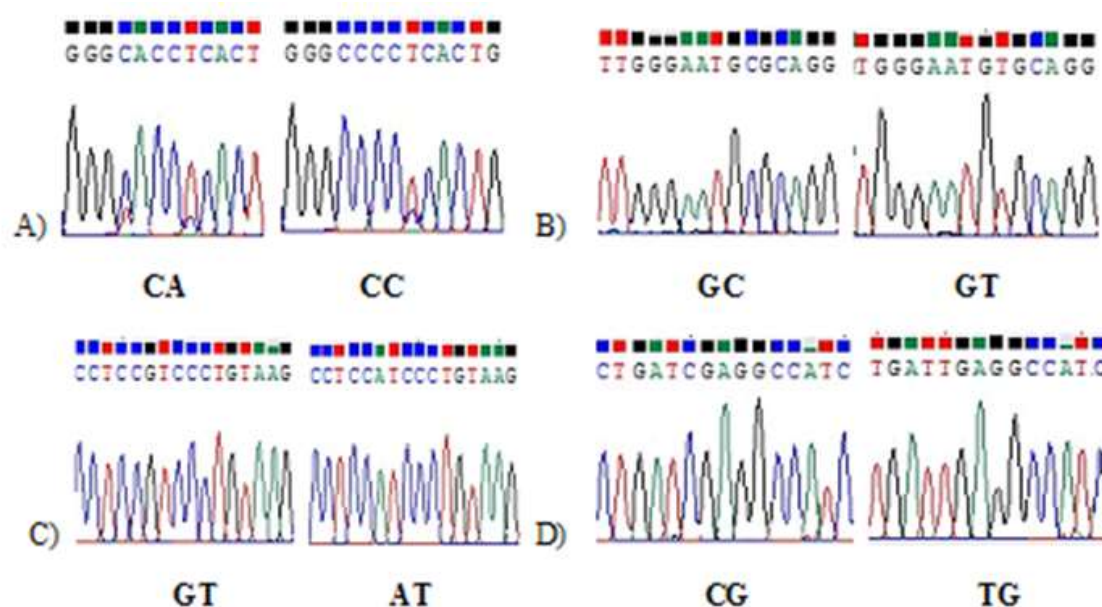| Genotype/allele	Case group (n=99)	Control group (n=100)	*P* value interval	95% Confidence
BsmI genotype (rs1544410)				
BB	62 (62.6%)	51 (51%)		
Bb	20 (20.2%)	29 (29%)		
Bb	17 (17.2%)	20 (20%)	0.347	0.699 (0.332-1.473)
BsmI allele				
B	144 (72.7%)	131 (70.5%)		
B	54 (27.3%)	69 (29.5%)		
FokI genotype (rs2228570)				
FF	32 (32.3%)	40 (40%)		
Ff	24 (24.2%)	36 (36%)		
ff	43 (43.4%)	24 (24%)	0.021	2.240 (1.132-4.431)
FokI allele				
F	88 (44.4%)	116 (58%)		
F	110 (55.6%)	84 (42%)		
TaqI genotype (rs731236)				
TT	27 (27.3%)	30 (30%)		
Tt	21 (21.2%)	31 (31%)		
tt	51 (51.5%)	39 (39%)	0.272	1.453 (0.746-2.829)
TaqI allele				
T	75 (37.9%)	91 (45.5%)		
T	123 (62.1%)	109 (54.5%)		
ApaI genotype (rs7975232)				
AA	44 (44.4%)	40 (40%)		
Aa	21 (21.3%)	28 (28%)		
aa	34 (34.3%)	32 (32%)	0.916	0.966 (0.507-1.842)
ApaI allele				
A	55.05%	108 (58%)		
A	44.95%	92 (46%)		

Fig. 1. Sequence analysis of DNA samples. A; The sequence of *ApaI* (C> A) polymorphism with A and C alleles, B; *BsmI* polymorphism with C and T alleles (sequenced with reverse primer), C; *FokI* polymorphism with G and A alleles (sequenced with reverse primer), D; TaqI polymorphism (T> C) with C and T alleles.

Discussion

In recent years, numerous studies have been conducted on the association between *VDR* gene polymorphism and various types of cancer, and it is hypothesized that the polymorphism of this gene might affect the risk of cancer development and its prognosis (20). Vitamin D receptor (VDR)is a high-affinity receptor that serves as a transcription factor upon ligand binding and activation. Ligand binding triggers its hetero dimerization with x retinoid receptor (21, 22). The activated high-affinity heterodimer binds to the vitamin D response elements located within the promoter regions of the target genes adjacent to the transcriptional machinery (22, 23). More than 60 different types of polymorphisms have been identified within this gene, most notably *FokI*, *BsmI*, *ApaI*, and TaqI (24). Various reports have been presented on the association of VDR polymorphisms with breast, skin, and prostate cancers (25).

FokI is located at the 5' end of the *VDR* gene. In this SNP, due to the nucleotide substitution of T to C within the first codon of exon 2 (ATG to ACG giving rise to the allelic conversion of "f" to "F"), the first translation initiation site (TIS) is eliminated ultimately resulting in the production of a peptide shorter than the wild type peptide (3 amino acids absent) hence creating a new form of VDR which is more transcriptionally active (25). Concerning this VDR polymorphism, we detected a higher frequency of "f" allele in gastric cancer patients in comparison to healthy individuals. In line with our finding, Cong et al. in 2015 found a higher frequency of "f" allele among gastric cancer patients compared to controls in a Chinese Han population (26). The TaqI polymorphism is located within exon 9, codon 352, in which a T nucleotide has been substituted with a C. Since VDR is a transcriptional regulating factor for a large number of target genes, its altered expression can influence various aspects of cellular function (27, 28). In this study, we found a higher frequency of TaqI polymorphism in gastric cancer patients. In a meta-analysis carried out by Serrano et al. in 2016 on the association of VDR polymorphisms with the risk of cancer, TaqI SNP was demonstrated to confer a 43% increased risk of

colorectal cancer (CRC)as another cancer of the gastrointestinal (GI) tract (29). In a study, TaqI polymorphism was shown to significantly associate with vulnerability to Crohn's disease with an elevated TaqI "t" allele frequency in these patients (30).

ApaI polymorphism, located within exon 9 adjacent to the 3' untranslated region (3' UTR), negatively regulates VDR expression. In this SNP, G is replaced by T (28, 31). *BsmI* is located within intron 8 and near the 3'UTR region and is generated as a result of nucleotide substitution of G to A. This polymorphism also affects VDR mRNA stability (25). According to our results, *ApaI* and *BsmI* SNPs did not associate with gastric cancer. The same finding was reported by Vidigal et al. that, a heterozygous genotype of *ApaI* SNP (Aa) or the association genotype (aa+Aa) predisposed to CRC as a GI cancer whereas *BsmI* SNP did not significantly associate with the risk of CRC (32). Martins et al. reported that *FokI*, *TaqI,* and *ApaI* polymorphisms were not associated with Helicobacter pylori (H. pylori) infection, while *BsmI* polymorphism showed a possible association with infection byH. Pylori (33). Kevin et al., in a case-control study reported that serum vitamin D levels in gastric incomplete intestinal metaplasia patients were lower compared with healthy subjects (34).

In conclusion, the results of this study demonstrated that *FokI* polymorphism is associated with gastric cancer risk. However, TaqI *ApaI* and *BsmI* polymorphisms are not associated with gastric cancer. However, future researches with a larger sample size are needed to confirm these results. This study can be used as a basis for studying other polymorphisms of VDR in association with gastric cancer.

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