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FokI Polymorphism of the Vitamin D Receptor Gene Is Associated with Susceptibility to Gastric Cancer: A Case-Control Study

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Vitamin D is a potential protective agent against cancer, and its activity is mediated mainly by vitamin D receptor (VDR). The FokI polymorphism (rs10735810) represents a T-to-C transition (ATG to ACG) in exon 2 of the VDR gene, and this ATG represents the translation-initiation codon, encoded by the f allele. The FokI polymorphism results in the generation of a protein shortened by three amino acids, translated from the downstream ATG codon (the F allele). We investigated the relationship between the FokI polymorphism and gastric cancer in a Chinese Han population. A total of 187 patients and 212 healthy controls were enrolled. The FokI polymorphism was detected by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis. The f allele frequency was higher in patients than that in controls (51.6% and 43.6%, $P < 0.05$). Multivariate logistics regression analysis revealed patients with the f allele (Ff + ff) showed a higher risk of gastric cancer [odds ratio (95% confidence interval) 2.73 (1.13~4.32)]. Patients with the f allele (Ff + ff) also presented a poorly differentiated type of gastric cancer ($P < 0.05$) and higher levels of C-reactive protein on admission than the FF group (5.5 ± 2.4 mg/L vs. 3.4 ± 1.3 mg/L, $P < 0.05$). Here, we show an association between the VDR FokI polymorphism and the susceptibility to gastric cancer, which may be helpful for early detection of high-risk individuals with the f allele for gastric cancer. Conversely, the F allele may be a protective factor against gastric cancer.

Keywords: C-reactive protein; gastric cancer; histological differentiation; polymorphism; vitamin D receptor
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Introduction

Vitamin D is a fat-soluble secosteroid involved in a variety of biological processes, including bone metabolism, cell proliferation and differentiation. There is an inverse relationship between serum vitamin D level and incidence of cancers (Mohapatra et al. 2013). By using solar ultraviolet-B (UV-B) exposure in the skin as an index of vitamin D3 photoproduction, recent studies have found a highly significant inverse association between UV-B and mortality in many kinds of cancer (Peterlik et al. 2009). Vitamin D has been shown to reduce proliferation and increase differentiation in human colon cancer, and a higher serum 25-OH vitamin D level has been shown to be associated with decreased colorectal adenoma risk (Uitterlinden et al. 2004). In addition, the incidences of colon, rectal, breast, endometrial, renal and ovarian cancers exhibit a significant inverse relationship to the oral intake of calcium (Peterlik et al. 2009).

Vitamin D activity is mediated by the vitamin D receptor (VDR), and the gene encoding VDR is known to have several polymorphisms. Among the VDR gene polymorphisms, the FokI polymorphism (rs10735810) is not in linkage disequilibrium with other polymorphic sites (Slattery et al. 2004). The FokI polymorphism represents a T-to-C transition (ATG to ACG) in exon 2 of the VDR gene, and the ATG encodes the translation-initiation codon of VDR mRNA (the T or f allele). This T-to-C transition results in the generation of a protein shortened by three amino acids (C or F allele), translated from the downstream ATG codon (Chen et al. 2001; Chen et al. 2005), while the T or f allele encodes a longer protein. This transition also results in the loss of the FokI-recognition site. Importantly, the short variant of VDR, encoded by the F allele, was shown to be more efficient for transactivating vitamin D-target genes (Arai et al. 1997). Several studies have indicated that the FokI polymorphism of the VDR gene is associated with many kinds of human cancers (Slattery 2007; Raimondi et al. 2009). However, the association between the FokI polymorphism and gastric cancer remains to be investigated.

Vitamin D has been implicated as a potential agent for the prevention of colorectal cancer through mechanisms mediated by the VDR (Park et al. 2006). Additionally, VDR gene polymorphisms have been suggested to be an

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important risk factor for colorectal cancer, either independently or in conjunction with vitamin D and calcium intake (Slattery et al. 2004). Our previous research demonstrated that VDR expression detected by immunohistochemistry in gastric tumor tissue was significantly lower than that in normal gastric mucosa, and well-differentiated carcinoma had the highest level of VDR expression (Miao et al. 2012). This suggests that VDR expression could be considered a differentiated marker of gastric cancer. In this case-control study, we investigated the possible relationship between the VDR-FokI polymorphism and the susceptibility to gastric cancer.

Materials and Methods

Participants

The study was carried out in the Department of Oncology of Shandong Provincial Hospital affiliated to Shandong University. A total of 187 gastric cancer patients who were admitted to Shandong Provincial Hospital from July 2009 to March 2014 and 212 healthy controls were enrolled during the same periods. All participants were required to be unrelated individuals of the Chinese Han population. The gastric cancer was identified by a pathological diagnosis at the department of Pathology of Shandong Provincial Hospital affiliated to Shandong University. The clinical staging of the gastric cancer was assessed according to the American Joint Commission on Cancer (AJCC) tumor-node-metastasis (TNM) staging system. The healthy individuals who were recruited from the Health Physical Examination Center of Shandong Provincial Hospital served as controls. Controls were frequency matched to cases by sex and age at enrollment (± 5 years). Each study participant signed a written informed consent for the interview and blood sample donation. A 5-mL venous blood sample was collected from each participant. All cases and controls completed a questionnaire to obtain information on demographic characteristics (age, sex, body mass index, and occupation), history of smoking and alcohol drinking, family history of cancer and frequency of sunshine exposure. Smokers were defined as those who smoked more than one cigarette/pipe per day for at least half a year, and drinkers were defined as those who consumed two or more alcoholic drinks per week for at least half a year. A family history of cancer referred the first- and second-degree relatives (parents, grandparents, siblings and offspring) of the study participants who had malignant tumors, especially gastric cancer (Huang et al. 2014). We recorded the baseline data of each participant in detail. We also recorded the results of biochemical and tumor related markers for the patients with gastric cancer. This study was performed in accordance with the ethical standards formulated in the Helsinki Declaration of 1964 and approved by the Shandong Provincial Hospital's Ethics Committee.

Genotyping

Genomic DNA was isolated by a commercial DNA isolation kit (Tiangen Biotech Co. Ltd., Beijing, China) according to the manufacturer's instructions. The VDR FokI genotype was analyzed using the PCR- RFLP method (Wilkinson et al. 2000; Liu et al. 2007). The forward primer was 5′-AGCTGGCCCTGGCACTGACTCTGC TCT-3′, and the reverse primer used was 5′-ATGGAAACACCT TGCTTCTTCTCCCTC-3′. The amplification was accomplished with a 50 μL reaction mixture containing 5 μL of 20 ng template DNA, 0.25 μL of each primer, 25 μL of 2 × PCR Master Mix (Tiangen Biotech Co. Ltd., Beijing, China). The PCR conditions were predenaturing at 94°C for 4 min, then 35 cycles (94°C for 45 s, 60°C for 45 s, and 72°C for 45 s), and a final extension at 72°C for 5 min. The PCR products were stored at 4°C. PCR products were digested with the FokI restriction endonuclease (New England Biolabs) at 37°C for 2 h. When the FokI restriction site was present (f genotype), the 265 bp fragment was digested into two fragments of 196 bp and 69 bp. The F genotype was not cleaved and had a single band of 265 bp, whereas the heterozygous (Ff) genotype showed bands of 265 bp, 196 bp and 69 bp. The polymorphism was then divided into three groups: excisable (ff), non-excisable (FF) and heterozygote (Ff).

Other information

Height and weight were measured at the time of interview. Body mass index (BMI) of weight/height^2 was calculated for the cases and controls. Information was also collected on regular use of aspirin and non-steroidal anti-inflammatory drug (NSAID). Sunshine exposure time referred to the average number of hours per week spent outside in the daytime. The results of biochemical markers including C-reactive protein (CRP) were acquired from the Central Laboratory of Shandong Provincial Hospital. The serum vitamin D levels were estimated by a chemiluminescence immunoassay. The TNM staging and the extent of histological differentiation of the gastric cancer were performed by the experienced oncologists and pathologists of Shandong Provincial Hospital.

Statistical analysis

Statistical analysis was performed by the SPSS17.0 statistical software package (SPSS Inc., Chicago, IL, USA). Values are expressed as mean ± SE. Univariate analysis for categorical variable was performed using the chi-squared test or Fisher's exact test as appropriate. Differences in numerical variables among the two groups were analyzed by an unpaired Student's *t*-test. Significant factors produced by the above methods were analyzed using multivariate logistical regression analysis. Logistic regression analysis was also used to calculate the odds ratios (ORs), 95% confidence intervals (CIs) and corresponding *P*-values. A *P*-value of < 0.05 was considered statistically significant.

Results

Gastric cancer and VDR FokI polymorphism

The Hardy-Weinberg equilibrium was evident for the gene polymorphism of VDR-FokI gene in both the case and control groups. The genotypes of FF, Ff and ff were 22.5%, 51.9%, 25.6% in gastric cancer cases (n = 187) and 32.1%, 48.6%, 19.3% in healthy controls (n = 212) respectively ($P = 0.07$). Subjects with the f allele (Ff + ff) showed a susceptibility to gastric cancer (77.5% and 67.9% respectively, $P < 0.05$). The f allele frequency in patients and controls were significantly different (51.6% and 43.6% respectively, $P < 0.05$). No significant differences were found between the two groups in age (> 60 years old, 69.5% vs. 70.3%), sex (male, 59.9% vs. 62.7%), BMI (23.1 ± 7.4 kg/m^2 vs. 22.9 ± 8.1 kg/m^2), smoking history (48.1% vs. 46.7%), alcohol consumption (43.9% vs. 39.6%), frequent NSAID intake (29.4% vs. 36.8%), and sunshine exposure time per day (1.7 ± 1.4 hours vs. 1.9 ± 1.7 hours). In addition, the frequency of a family history of tumors was higher in the

Table 1. Baseline clinical characteristics of the study participants.[a]

	Gastric cancer cases (n = 187)	Controls (n = 212)	*P*-value
Age (> 60 yrs, %)	130 (69.5)	149 (70.3)	NS
Sex (Male, %)	112 (59.9)	133 (62.7)	NS
BMI (kg/m^2)	23.1 ± 7.4	22.9 ± 8.1	NS
Family history of tumors (%)	28 (14.9)	12 (5.7)	< 0.05
Smoking history (%)	90 (48.1)	99 (46.7)	NS
Alcohol consumption (%)	82 (43.9)	84 (39.6)	NS
Frequent NSAID intake (%)	55 (29.4)	78 (36.8)	NS
Sunshine exposure (hours/day)	1.7 ± 1.4	1.9 ± 1.7	NS
Genotype			
FF (n, %)[b]	42 (22.5)	68 (32.1)	0.07[c]
Ff (n, %)	97 (51.9)	103 (48.6)	
ff (n, %)	48 (25.6)	41 (19.3)	
Ff + ff (n, %)	145 (77.5)	144 (67.9)	< 0.05
f allele (n, %)	193 (51.6)	185 (43.6)	< 0.05

BMI, body mass index; NSAID, non-steroidal anti-inflammatory drug; NS, not significant.
[a]Subjects with the f allele (Ff + ff) showed a susceptibility to gastric cancer.
[b]Controls vs. cases, $P < 0.05$.
[c]Compared with FF, Ff and ff genotypes of cases and controls.

Table 2. Adjusted odds ratio (OR) and 95% confidence interval (CI) for susceptibility of gastric cancer with f allele (Ff + ff) according to FF genotype by logistics regression.[a]

Genotype	Cases (%)	Controls (%)	OR (95% CI)	*P*-value
FF (n, %)	42 (22.5)	68 (32.1)	1.00 (referent)	
Ff + ff (n, %)	145 (77.5)	144 (67.9)	2.73 (1.13~4.32)	< 0.05

[a]Subjects with the f allele (Ff + ff) revealed a higher risk of gastric cancer than the subjects without the f allele, after adjusting for age, sex, BMI, family history of tumors, smoking history, alcohol consumption, frequent NSAID intake and sunshine exposure time.

case group than the control group (14.9% vs. 5.7%, $P < 0.05$) (Table 1).

A multivariate logistic regression analysis showed that subjects with the f allele (Ff + ff) revealed a higher risk of gastric cancer than the subjects without the f allele (FF), after adjusting for confounding factors (age, sex, BMI, family history of tumor, smoking history, alcohol consumption, frequent NSAID intake, and sunshine exposure time) (OR = 2.73, 95% CI 1.13~4.32, $P < 0.05$) (Table 2).

Differentiation of gastric cancer and CRP

Among the 187 patients with gastric cancer, 42 (22.5%) exhibited the FF genotype, 97 (51.9%) were heterozygous with the Ff genotype, and 48 (25.6%) were homozygous for the ff genotype. We divided the patients into two groups: patients without the f allele (FF, n = 42), and patients with the f allele (Ff + ff, n = 145). A worse histological feature (poorly rather than moderately or well differentiated) was shown in the Ff + ff group than that in the FF group (52.4%, 34.5%, and 13.1% vs. 33.3%, 40.5%, and 26.2% respectively, $P < 0.05$). In addition, the CRP level on admission was higher in the Ff + ff group compared with the FF group (5.5 ± 2.4 mg/L vs. 3.4 ± 1.3 mg/L, $P < 0.05$).

Other biochemical markers and VDR FokI polymorphism

There were no statistical differences between the two groups in the serum concentration of total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), CEA, CA-125, uric acid, and AJCC TNM staging (stage I to IV) ($P > 0.05$) (Tables 3 and 4). The serum vitamin D and calcium levels also showed no statistical difference between the patients with or without the f allele (Table 3).

Discussion

In this study, we investigated the relationship between the VDR FokI gene polymorphism and the susceptibility to gastric cancer in a Chinese Han population. The F allele of the VDR FokI may be a protective factor against gastric cancer. Compared with the subjects without the f allele (FF), the risk of gastric cancer increased 2.73-fold in subjects with the f allele (FF + Ff). There was no difference between the case and control groups for frequent NSAID

Table 3. Plasma levels of biochemical markers of the patients with or without the f allele.[a]

	FF (n = 42)	Ff + ff (n = 145)	*P*-value
Lipid			
TC (mmol/L)	4.7 ± 1.1	4.6 ± 1.2	NS
TG (mmol/L)	1.9 ± 1.0	1.8 ± 1.4	NS
LDL-C (mmol/L)	2.8 ± 0.4	3.0 ± 0.3	NS
HDL-C (mmol/L)	1.9 ± 0.4	1.7 ± 0.6	NS
CRP[b] (mg/L)	3.4 ± 1.3	5.5 ± 2.4	< 0.05
uric acid (μmol/L)	215 ± 34.3	222 ± 36.5	NS
calcium (mmol/L)	2.4 ± 0.3	2.3 ± 0.2	NS
vitamin D (ng/mL)	27 ± 5.6	25 ± 7.8	NS

TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; CRP, C-reactive protein; NS, not significant.
[a]There were no differences of the biochemical markers levels between the two groups other than CRP.
[b]CRP level was tested on admission.

Table 4. Tumor markers and staging of the patients with or without the f allele.[a]

	FF (n = 42)	Ff + ff (n = 145)	*P*-value
Tumor markers			
CEA (ng/mL)	3.3 ± 1.6	3.7 ± 1.8	NS
CA-125 (U/mL)	9.4 ± 4.6	10.8 ± 6.2	NS
TNM Staging			
Stage I and II (n, %)	12 (28.6)	39 (26.9)	NS[b]
Stage III (n, %)	17 (40.5)	57 (39.3)	
Stage IV (n, %)	13 (30.9)	49 (33.8)	
Histological differentiation			
Well (n, %)	11 (26.2)	19 (13.1)	< 0.05[c]
Moderately (n, %)	17 (40.5)	50 (34.5)	
Poorly (n, %)	14 (33.3)	76 (52.4)	

CEA, carcino-embryonic antigen; CA-125, cancer antigen 125; NS, not significant.
[a]The TNM staging and histological differentiation of the gastric cancer were performed by the experienced oncologist and pathologists.
[b]Compared with stage I+II, III and IV of the two groups.
[c]Compared with well, moderately and poorly differentiated types of the two groups.

intake or sunshine exposure time, but the subjects with a family history of tumors in case group was more than that in control group, which indicate that the FokI polymorphism may affect the genetic susceptibility to gastric cancer independently of the environmental agents.

It has been previously demonstrated that vitamin D acted as a prognostic indicator of gastric cancer and may be correlated with the incidence risk of gastric cancer (Bao et al. 2013). A possible mechanism that has been suggested was the increase of apoptosis induced by the active metabolite of vitamin D3 (Diaz et al. 2000). The available data suggest that vitamin D level may influence the development of many cancers (Lappe et al. 2007). The VDR is an intracellular hormone receptor that binds to the biologically active form of vitamin D, 1, 25-dihydroxyvitamin D3 or calcitriol. It produces a variety of biologic effects by interacting with specific nucleotide sequences of target genes. The VDR is known to be a crucial mediator of the cellular effects of vitamin D and interacting with cell signaling pathways to influence cancer development (Kearney et al. 1996; Deeb et al. 2007; Ishihara et al. 2008; Theodoratou et al. 2008; Tang et al. 2009).

The VDR gene is located on chromosome 12q12-q14 and several single-nucleotide polymorphisms (SNP) have been identified that may influence cancer risk (Raimondi et al. 2009). Recent studies have indicated the polymorphisms of the VDR gene, but the influence of these polymorphisms on VDR protein function and signaling is still unknown (Wilkinson et al. 2000; Park et al. 2006; Raimondi et al. 2009). Most studies have focused on these polymorphisms: 1) rs7975232 or ApaI on intron 8, 2) rs1544410 or BsmI on intron 8, 3) rs10735810 or FokI polymorphism on exon 2, 4)

rs731236 or TaqI on exon 9, 5) rs757343 or Tru9I on intron 8, and 6) the poly (A) mononucleotide repeat at the 3′-UTR region of the gene. These polymorphisms of VDR may have different functions depending on their locations (Slattery 2007). The FokI polymorphism is located near the 5′-UTR region of the gene within the DNA-binding domain (Kanan et al. 2000; Ingles et al. 2001; Kim et al. 2001; Chen et al. 2002; Tworoger et al. 2009). Studies have explored the associations between VDR polymorphisms and human cancers, including colorectal adenomas, prostate cancer, breast cancer, bladder cancer and melanoma (Kearney et al. 1996; Deeb et al. 2007; Slattery 2007; Ishihara et al. 2008; Theodoratou et al. 2008; Tang et al. 2009).

The FokI polymorphism changes the first potential start codon in the VDR gene from ATG to ACG (the f allele encodes ATG at the translation start site, and the F allele encodes ACG at the same site), resulting in a VDR protein that is shorter by three amino acids, designated the F variant. The F variant was shown to be more efficient for transactivating vitamin D-target genes (Arai et al. 1997). The present study also suggests that the F allele of VDR FokI may be a protective factor against gastric cancer. The protective effect of the F allele was expected to provide stronger anti-proliferative and pro-differentiation signals to human cancers (Wong et al. 2003).

Gastric cancer is the fourth most common cancer and the second leading cause of cancer-related deaths worldwide and the second most common cancer and the third most common cause of death in cancer patients in China (Yuan et al. 2012; Huang et al. 2014). However, only 10% of patients were diagnosed at an early stage in China, and the other patients diagnosed at an advanced stage (TNM III or IV). The tumor is inoperable and the five-year survival rate is less than 10% (Yu et al. 2013). Gastric cancer is a multifactorial disease that results from multiple exposures to environmental factors, life-style risk factors, and individual genetic predisposition. Early detection by gene detecting of the high-risk population is an important way to improve the prognosis of gastric cancer. Our study showed the F allele of VDR gene may play a protective role against gastric cancer. And people with f allele performed the susceptibility to gastric cancer should be early intervention to prevent the occurrence of gastric cancer.

CRP is widely recognized as a sensitive but nonspecific systemic marker of inflammation involved in host defense (Sasazuki et al. 2010; Lukaszewicz-Zając et al. 2011). Recently, CRP had also been shown to have a prognostic value in cancer (Yu et al. 2013). An increased pretreatment serum CRP level had been shown to be significantly associated with poor prognosis for gastric cancer patients, either in early or advanced stages, and the progression-free survival (PFS) was significantly shorter in high-CRP level patients (Yu et al. 2013). This study revealed that the serum CRP level of the patients with the f allele (Ff + ff) was higher than the patients without the f allele, indicating more severe inflammation conditions and worse prognosis of gastric cancer.

Our previous research suggested that VDR expression could be considered as a differentiated marker of gastric cancer, and well-differentiated carcinoma performed the highest level of VDR expression (Miao et al. 2012). In this study the patients without f allele (FF genotype) were associated with a better histological differentiation of gastric cancer (well- or moderately differentiation). As it has been reported that the extent of vitamin D-dependent transcriptional activation under the control of a vitamin D response element in vitro was approximately 1.7-fold greater for the F allele than for the f allele protein (Arai et al. 1997), one of the possible reason was that a high-efficiency VDR may favorably mediate the anti-proliferative and pro-differentiation role of vitamin D to play a protective role on gastric cancer.

This study has important strengths: it was a case-control study and reported the association between the FokI polymorphism of VDR gene and gastric cancer along with histological differentiation and CRP levels. However, there are also several limitations. First, it was a small-scale, single-center study, and so the data may not be universally applicable. Further studies should be conducted in different populations. Another limitation was that the mechanism of gene polymorphisms and the inflammatory environment in a different differentiated-type individual was still unknown. It would be needed to confirm the molecular mechanisms and consider this interaction.

In conclusion, our findings indicate that the VDR FokI polymorphism is associated with the susceptibility to gastric cancer. Patients with the f allele showed higher levels of CRP and worse histological differentiation, indicating poor prognosis of gastric cancer. The f allele may be a high-risk allele for gastric cancer, which may be helpful in early detection of patients at high risk of gastric cancer. Additional studies are needed to confirm the molecular mechanisms involved.

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Conflict of Interest

The authors declare no conflict of interest.

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