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Vitamin D receptor genetic variants are associated with susceptibility of gallbladder adenocarcinoma in a Chinese cohort

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ABSTRACT. The aim of this study was to test for the possible association between vitamin D receptor (VDR) genetic variants and susceptibility to gallbladder cancer (GBC). A total of 291 GBC cases were recruited and 396 gender- and age-matched healthy volunteers were enrolled as controls. The *VDR* gene polymorphisms were determined in all subjects. The genotype and the allele frequencies of *Apa*I, *Bsm*I, and *Taq*I polymorphisms were not significantly different between GBC subjects and controls. However, the genotype and allele frequencies of the *Fok*I C>T polymorphism were significantly different between GBC subjects and controls. The *Fok*I TT genotype was in markedly higher frequency in GBC subjects compared to controls (38.14 *vs* 22.73%, $P < 0.001$). Using TT as the reference genotype, multivariate logistic regression analysis showed that CC genotype carriers had a higher risk of GBC (adjusted odds ratio (OR) = 3.423, adjusted $P = 0.001$) with adjustment for age, gender, smoking status, alcohol use, and gallstone presence, as well as the serum 1,25(OH)2D level. Carriers of the CT genotype also had a higher risk of GBC (adjusted OR = 1.992, adjusted

P = 0.003). Multivariate logistic regression analysis did not reveal any association between the *Apa*I, *Bsm*I, and *Taq*I polymorphisms and GBC risk (all P > 0.05).

Key words: Vitamin D receptor; Gallbladder cancer; Susceptibility

INTRODUCTION

Gallbladder cancer (GBC) is one of the most common malignant neoplasms of the digestive tract (Erickson and Nag, 1998). GBC has a multifactorial etiology including gender, obesity, chronic cholecystitis, gallstones, and occupational exposure to specific chemicals (Moerman et al., 1997; Lowenfels et al., 1999; Pandey and Shukla, 2002; Serra et al., 2002). Previous studies have shown that genetic factors are also involved in the development of GBC in several populations (Zatonski et al., 1997; Roa et al., 2000; Pitt and Brenner, 2003; Qiu et al., 2005; Kim et al., 2008). Several genetic polymorphisms have been reported as potential molecular markers for GBC (Srivastava et al., 2008a,b, 2010). However, ideal candidate gene prediction of GBC risk is still under investigation.

Epidemiological studies indicate that vitamin D insufficiency could play an etiological role in various human cancers (Peterlik et al., 2009; Garland et al., 2009; Trump et al., 2009; Hines et al., 2010; Mitchell, 2011). The effect of vitamin D is mediated by the vitamin D receptor (VDR), a member of the nuclear receptor superfamily of ligand-inducible transcription factors, which are involved in many pathological processes (Subramaniam et al., 2001; Kato and Yoko, 2004; Slattery, 2007). Several single-nucleotide restriction fragment length polymorphisms (RFLPs) have been described in the *VDR* gene in association with carcinogenesis. Increasing studies have revealed that *VDR* gene polymorphisms influence the risk of certain cancers, including breast, prostate, skin, colon-rectum, bladder, and liver cancer, as well as renal cell carcinoma and malignant melanoma (Slattery et al., 2001, 2007; Han et al., 2007; Yaylim-Eraltan et al., 2007; Li et al., 2008; Kostner et al., 2009). However, the role of *VDR* gene polymorphisms in determining the risk for GBC remains undocumented. In the present study, we performed a case-control analysis in Chinese cohorts to test the possible association between *VDR* genetic variants and GBC.

MATERIAL AND METHODS

Study subjects

We enrolled 291 patients with histologically confirmed gallbladder adenocarcinoma from March 2003 to December 2010 at our hospital. The exclusion criteria were as follows: occupational exposure to ultraviolet radiation or chemical carcinogens, chronic bacterial infections, typhoid carrier, and ulcerative colitis. A further 396 gender- and age-matched healthy volunteers were enrolled as controls. Informed consent, blood samples, and clinical evaluations were collected from all subjects according to protocols that were approved by the Institution Review Boards of the Ethics Committees of all of the participating institutes.

Sample collection and genotyping

Ten milliliters of venous blood was collected from each patient into tubes containing 50 mM ethylenediaminetetraacetic acid (EDTA), and genomic DNA was isolated with the DNA Blood Mini kit, according to manufacturer instructions (QIAGEN, Shanghai, China). Four diallelic polymorphisms of *VDR* were genotyped: The *Fok*I C>T (rs10735810) and *Taq*I T>C (rs10735810) polymorphic sites on the coding sequence, and the *Bsm*I A>G (rs1544410) and *Apa*I G>T (rs7975232) polymorphisms on the last intron. The polymerase chain reaction (PCR) technique was applied followed by RFLP assays. The primers and conditions for the *VDR* polymorphisms are listed in Table 1. All PCR products were identified by electrophoresis on 2% agarose gel stained with ethidium bromide.

Table 1. PCR-RFLP primers and conditions for *VDR* gene polymorphisms.

SNP	Primer	Base change	T (°C)
*Fok*I	F: 5'-AGCTGGCCCTGGCACTGACTCTGCTCT-3' R: 5'-ATGGAAACACCTTGCTTCTTCTCCCTC-3'	C/T	61
*Bsm*I	F: 5'-CAACCAAGACTACAAGTACCGCGTCAGTGA-3' R: 5'-AACCAGCGGGAAGAGGTCAAGGG-3'	G/A	57
*Apa*I	F: 5'-CAGAGCATGGACAGGGAGCAA-3' R: 5'-GCAACTCCTCATGGCTGAGGTCTC-3'	G/T	60
*Taq*I	F: 5'-CAGAGCATGGACAGGGAGCAA-3' R: 5'-GCAACTCCTCATGGCTGAGGTCTC-3'	T/C	60

Serum levels of 1,25(OH)2D

The serum levels of 1,25(OH)2D were measured in a 25(OH)2D3-Elecsys vitamin D3 chemiluminescent immunoassay system (Roche Diagnostics, Mannheim, Germany) by using a photomultiplier (Elecsys 2010; Hitachi, Tokyo, Japan).

Statistical analyses

The χ^2 test was used to compare genotype frequencies and demographic distributions between cases and controls. Multiple logistic regression analysis was used to evaluate whether each polymorphism was independently associated with GBC when adjusted for the potential confounding effects of important clinical variables. The odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. The associations between *VDR* haplotypes and GBC risk were analyzed. Linkage disequilibrium (D' value) of the 4 single-nucleotide polymorphisms (SNPs) studied was calculated with the SHEsis software (Shi and He, 2005). The differences in overall survival (OS) and progression-free survival (PFS) across different genotypes were compared using the log-rank test with adjustments for age, gender, smoking status, cancer stage, differentiation status, and chemotherapy regimens. A Cox regression model was performed to obtain the adjusted hazard ratio (HR) and 95%CI of potential prognostic factors for OS in GBC patients. $P < 0.05$ was considered to be statistically significant. All analyses were conducted with the Statistical Package for the Social Sciences (SPSS version 16.0; SPSS Inc., Chicago, IL, USA).

RESULTS

The clinical characteristics of GBC cases and controls are listed in Table 2. There were more smokers, alcohol users, and gallstones in the GBC group than in the control group ($P < 0.05$). The GBC group had a significantly lower mean serum 1,25(OH)2D level compared to controls ($P < 0.001$).

Table 2. Clinical characteristics of gallbladder cancer (GBC) cases and controls.

Characteristic	Controls	GBC	P
Gender (female, %)	66.62	67.96	0.452
Age (years)	51.73 ± 6.24	51.41 ± 8.18	0.569
BMI (kg/m^2)	22.8 ± 2.3	22.1 ± 1.4	0.053
Tobacco users (%)	20.7	36.60	0.011
Alcohol users (%)	18.45	25.58	0.004
Gallstone presence (%)	9.63	36.67	<0.001
Serum 1,25(OH)2D (ng/mL)	34.3 ± 4.6	22.4 ± 6.7	<0.001

Data are reported as means ± SD, unless otherwise explained.

The genotype frequencies of *VDR* polymorphisms in GBC subjects and controls were found to be in Hardy-Weinberg equilibrium (all $P > 0.05$). The genotype and allele frequencies of *Apa*I, *Fok*I, *Bsm*I, and *Taq*I in cases and controls are shown in Table 3. The genotype and allele frequencies of *Apa*I, *Fok*I, *Bsm*I, and *Taq*I were not significantly different between GBC and controls. However, the genotypes and allele frequencies of the *Fok*I C>T polymorphism were significantly different between GBC subjects and controls. GBC subjects had a markedly higher *Fok*I TT genotype frequency compared to controls (38.14 *vs* 22.73%, $P < 0.001$). Using the TT genotype as reference, multivariate logistic regression analysis showed that CC genotype carriers had a higher risk of developing GBC (adjusted OR = 3.423, adjusted P = 0.001) with adjustment for age, gender, smoking status, alcohol use, and gallstone presence as well as the serum 1,25(OH)2D level. The CT genotype also represented a higher risk for GBC (adjusted OR = 1.992, adjusted P = 0.003). However, multivariate logistic regression analysis did not reveal any association between the *Apa*I, *Bsm*I, and *Taq*I polymorphisms and GBC risk (all $P > 0.05$; Table 3).

VDR haplotypes and GBC risk

The associations between the *VDR* haplotypes and the GBC risk were analyzed in this study. The D' values of the 4 SNPs analyzed were calculated with the SHEsis software. All 4 SNPs were in strong linkage disequilibrium (all $D' > 0.8$). The estimated haplotype frequencies of the *VDR* SNPs are shown in Table 4. The haplotypes C_{Fok}-A_{Bsm}-G_{Apa}-T_{Taq}, C_{Fok}-G_{Bsm}-T_{Apa}-T_{Taq}, and C_{Fok}-G_{Bsm}-T_{Apa}-C_{Taq} showed a significantly higher risk of developing GBC (OR = 4.967, 2.463, and 1.886, respectively, all $P < 0.05$; Table 4).

We further analyzed the variation of serum 1,25(OH)2D levels in GBC according to *VDR* genotype distributions. *Fok*I CC genotype carriers had significantly lower 1,25(OH)2D levels (CC: 17.5 ± 3.2 *vs* TC: 23.2 ± 1.7 and TT: 23.5 ± 2.4, both $P < 0.05$; Figure 1). When stratified by the *Apa*I, *Bsm*I, and *Taq*I polymorphisms, there were no differences among genotype carriers (data not shown).

Table 3. Genotype and allele frequencies of *Apa*I, *Fok*I, *Bsm*I, and *Taq*I between gallbladder cancer (GBC) cases and controls.

		GBC	N = 291	Control	N = 396	Adjusted OR	95%CI	Adjusted P value
		N	%	N	%			
*Apa*I	TT	83	28.52	104	26.26	1.000		
	TG	125	42.96	197	49.75	0.795	0.552-1.146	0.218
	GG	83	28.52	95	23.99	1.095	0.725-1.653	0.667
	T	291	50.00	405	51.14	1.000		
	G	291	50.00	387	48.86	1.047	0.845-1.296	0.677
*Bsm*I	AA	72	24.74	108	27.27	1.000		
	AG	176	60.48	210	53.03	1.257	0.878-1.801	0.211
	GG	43	14.78	78	19.70	0.827	0.513-1.332	0.051
	A	320	54.98	426	53.79	1.000		
	G	262	45.02	366	46.21	0.953	0.769-1.181	0.660
*Fok*I	TT	40	13.75	111	28.03	1.000		
	CT	140	48.11	195	49.24	1.992	1.307-3.037	0.003
	CC	111	38.14	90	22.73	3.423	2.169-5.400	0.001
	T	220	37.80	417	52.65	1.000		
	C	362	62.20	375	47.35	1.830	1.471-2.275	0.000
*Taq*I	TT	90	30.93	112	28.28	1.000		
	TC	156	53.61	198	50.00	0.980	0.692-1.388	0.912
	CC	45	15.46	86	21.72	0.651	0.413-1.026	0.054
	T	336	57.73	422	53.28	1.000		
	C	246	42.27	370	46.72	0.835	0.673-1.036	0.101

Table 4. Haplotype analysis of the VDR gene and gallbladder cancer (GBC) status

FokI	*Bsm*I	*Apa*I	*Taq*I	GBC (N)	Controls (N)	P	Odds ratio (95%CI)
C	A	G	T	97	15	<0.001	4.967 (2.873-8.586)
C	G	T	C	84	31	<0.001	2.463 (1.807-3.698)
C	G	T	T	73	75	0.034	1.866 (1.496-2.9737)

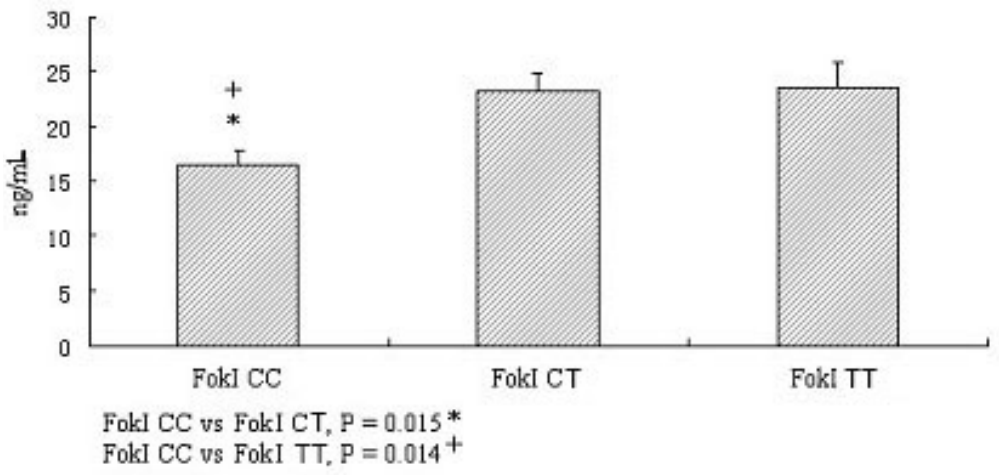

Figure 1. Serum 1,25(OH)2D in gallbladder cancer according to *Fok*I genotype distributions.

DISCUSSION

In the present hospital-based study, we investigated the possible association between polymorphisms of the *VDR* gene and GBC risk. We observed that carriers of the *Fok*I CC and TC genotypes had markedly increased ORs for GBC, suggesting that the C allele is a risk factor to GBC incidence. The *Fok*I C>T polymorphism also affected the serum 1,25(OH)2D levels. The polymorphisms of the other three loci, *Apa*I, *Bsm*I, and *Taq*I, did not show an association with GBC. To our knowledge, our study is the first to report the interaction between *VDR* polymorphisms and the risk of GBC.

The *Fok*I RFLP, located in the coding region of the *VDR* gene, results in the production of a VDR protein that is three amino acids longer than the wild-type. Although no significant differences in ligand affinity, DNA binding, or transactivation activity have been found between these two VDR forms when studied independently (Laaksonen et al., 2004), in transient transfection assays with a vitamin D-responsive reporter gene, the shorter VDR variant displayed higher potency than the longer one (Gross et al., 1998). It has been hypothesized that a less active VDR could be associated with either an increased susceptibility to cancer risk or to a more aggressive form of the disease (Kanan et al., 2000). In this study, we found that the *Fok*I polymorphisms of the *VDR* gene were closely associated with GBC risk, suggesting that this locus could be used as a molecular marker for GBC.

Both *in vitro* and *in vivo* studies have shown proapoptotic and anticancer effects upon binding of 1,25-dihydroxyvitamin D to the VDR in many different types of cancers (Reichel et al., 1989). Berger et al. (1988) demonstrated a dose-dependent decrease in the growth rate of melanoma cells treated with 1,25(OH)2D. The growth-inhibitory property of 1,25(OH)2D has since been reported in tumor-derived cells from other tissues, including the colon (Platz et al., 2000), breast (Mawer et al., 1997), and prostate (Platz et al., 2004; Tseng et al., 2009). In this study, we found that the 1,25(OH)2D levels were significantly different among different *Fok*I genotype carriers, suggesting that the effect of *Fok*I polymorphisms of the *VDR* gene on GBC risk might be mediated through the influence on 1,25(OH)2D levels.

Some limitations of this study need to be addressed. First, because this was a hospital-based study, there is the possibility of enrollment bias. Second, we only enrolled GBC patients in a Chinese cohort; therefore, whether the positive roles of *VDR* polymorphisms we found in this study also hold in other ethnic populations remains unknown. Third, we did not perform functional analyses to further determine the mechanism under which *VDR* polymorphisms affect the susceptibility of GBC.

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