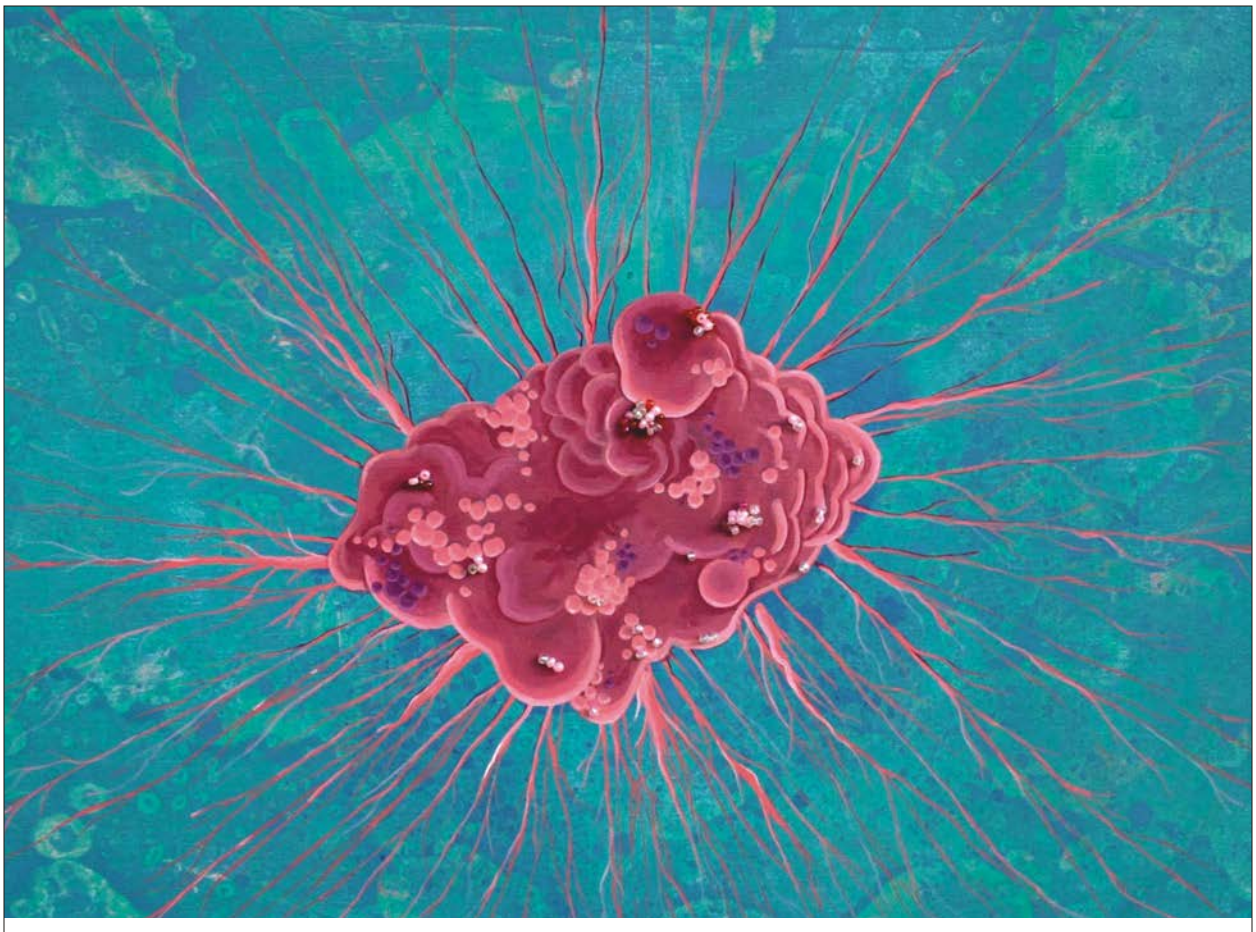

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Research Article
Vitamin D Receptor Gene Polymorphisms as a Predictive Risk Factor for Hepatocellular Carcinoma Development and Severity in Chronic Hepatitis B

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Abstract

Background and Objective: Vitamin D, considered as a systemic hormone and important immune modulator, has emerging roles in fibrogenesis, cell cycle arrest and cancer development. The objective of this study was to investigate the association between the Vitamin D Receptor (VDR) gene polymorphisms and hepatocellular carcinoma (HCC) development and severity in Egyptians with Chronic Hepatitis B (CHB). **Methods:** Two hundred and eighty-five adult consecutive outpatients were enrolled, of which 108 patients with CHB and HCC, 92 patients with CHB without HCC and 85 patients with HCC without CHB. Evaluation of clinicopathological characteristics of HCC was done. Genotyping of VDR gene at TaqI, BsmI, ApaI and FokI was also performed. **Results:** FokI TT genotype prevalence and T allele frequency were higher in HCC patients than those without HCC. Patients carrying FokI TT genotype had significantly higher risk for HCC ($p < 0.05$) after using FokI CC genotype as a reference and adjusting other covariates including age, gender, CHB infection time, family history of cancer and serum ∞-fetoprotein levels. Also, patients carrying FokI TT genotype had advanced disease stage of cancer, liver cirrhosis, lymph node metastasis. **Conclusion:** Only the SNPs of VDR gene at FokI locus (C>T) could be used as a molecular marker predicting the risk and evaluating the severity of HCC in Egyptian patients infected with CHB. Polymorphism at FokI locus was associated with increased HCC susceptibility and has a significant role in the determination of its clinicopathological characteristics.

Key words: Hepatocellular, carcinoma, vitamin D, polymorphisms, chronic hepatitis

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Vitamin D, an important immune modulator, has emerging roles in fibrogenesis, inflammatory liver diseases, cell cycle arrest and cancer development[1]. Vitamin D exerts its genomic effects by binding to Vitamin D Receptor (VDR) which is considered an endocrine member of nuclear receptor superfamily[2,3]. It has been established that there is an association between vitamin D deficiency and many health problems (e.g., Cancer development of various types, viral hepatitis)[4]. Also, the deficiency of vitamin D has been correlated with advanced disease stages[5]. Mohammed *et al.*[6] demonstrated that low vitamin D level in patients with chronic hepatitis B viral infection may be an indicator of the viral replication and predicts bad prognosis. Also, it has been well established that vitamin D exerts anti-proliferation, anti-angiogenesis, pro-differentiation and pro-apoptosis actions on many cancer cells expressing VDR[7].

Previous epidemiological studies showed that individual's susceptibility to cancer is mediated by a variety of genetic factors[8]. Epidemiologically, the association of vitamin D and HCC is still inconclusive; however, there are biochemical evidences that clearly indicate the response of HCC cells to the inhibitory effect of vitamin D and its analogs[9,10].

The carcinogenesis of hepatocellular carcinoma, one of the most common malignancies worldwide, is a complex, multistep and multifactorial process[11]. There are multiple risk factors contributing to hepatic carcinogenesis and HCC development, including chronic Hepatitis C Virus (HCV) or Hepatitis B Virus (HBV) infection, liver cirrhosis, carcinogen exposure and chronic alcoholism[12].

Also, it has been reported that several Single Nucleotide Polymorphisms (SNPs) in the VDR gene are associated with tumorigenesis in various organs such as prostate, breast, skin, colon, rectum and kidney but these observations are conflicting[13]. Moreover, VDR gene polymorphisms have been described in chronic liver diseases such as autoimmune hepatitis and primary biliary cirrhosis[14]. It has been reported that polymorphisms of VDR gene were associated with high occurrence of HCC in alcoholic liver cirrhosis in Caucasian subjects[15]. However, the data in literature describing the possible association between VDR the gene polymorphisms and HCC development are so far limited. Hence, the aim was to investigate the association between polymorphisms of VDR gene and HCC susceptibility and to assess its role in determining the disease severity in Egyptian patients with CHB.

MATERIALS AND METHODS

Patients: A total of 285 adult consecutive outpatient Egyptian subjects attending Department of Internal Medicine at Mansoura University Hospital from April 2014 through 2016 were initially enrolled in this study. The age range was 18-70 years. Female to male ratio was 0.926. Two hundred patients had CHB of which 108 patients had CHB with HCC and 92 patients had CHB with no HCC. Another eighty-five age-and sex-matched HCC patients with no CHB were also enrolled in this study. The study was approved by the Ethical Commission and Institutional review board of Mansoura Faculty of Medicine in Egypt (MFM-IRB; code No: R/16.02.50). A written informed conscious consent was obtained from all participants before their participation. The inclusion criterion was the diagnosis of chronic hepatitis B and/or HCC. Exclusion criteria were an age below 18 years and over 70 years, a history of cancer of any type within the last 5 years, a history of solid organ transplantation or previous bone marrow transplantation, antiviral treatment and local or systemic tumor-specific treatment within the last month. Patients with chronic renal failure, bone disorders, thyroid disorders, intestinal malabsorption, previous gastrectomy, OMMIT: hypovitaminosis D, taking vitamin D or antidepressant drugs, cardiac failure (ejection fraction <50%), chronic hepatitis C, hepatitis D, HIV infection, systemic bacterial or fungal infection, alcohol consumption, primary biliary cirrhosis, metabolic liver disease, non-alcoholic steatohepatitis and autoimmune hepatitis, were excluded from the present study.

Methods: Initially, all patients completed a detailed questionnaire regarding diet and habits and submitted to thorough history taking with detailed physical examinations and relevant medical history including cancer stage, lymph node metastasis and portal vein invasion[11]. Four milliliters of venous blood were obtained from all patients and control subjects and the serum samples were centrifuged at 3000 rpm then aliquoted and stored at -70°C until assayed. The model of end-stage liver disease (MELD) score[16], Child-Pugh score[17], serum α-fetoprotein, liver function tests, vitamin D level, ultrasound, CT scan, MRI and liver biopsies (if present) were assessed at inclusion time in this study. Also, 25 hydroxy-Vitamin D3 (25-OHD) levels were measured using a 25OH-Vitamin D3-direct ELISA Kit.

HCC was diagnosed by 4-phase multi-detector Computed Tomography (CT) scan, dynamic contrast-enhanced Magnetic Resonance Imaging (MRI). The HCC was confirmed if there is

typical features of dynamic imaging (arterial phase hypervascularity, delayed phase washout), liver nodule(s) of more than 1 cm in a cirrhotic liver or histopathological features of tumor tissues. Diagnosis of HCC was confirmed if there is one of the following three items[18,19]:

- One or more liver nodules >1 cm in diameter in CT or MRI
- Early arterial enhancement with α-fetoprotein $\geq$400 ng mL^{-1}
- Typical features of dynamic imaging regardless α-fetoprotein

Liver cirrhosis was diagnosed by ascites, esophageal varices, fundic varices, splenomegaly, jaundice, imaging and liver biopsies (if available, according to modified knodell histologic activity index)[20].

Chronic hepatitis B was diagnosed by positive HBs-Ag, negative anti-HBs for at least six months and positive HBV DNA levels done by Polymerase Chain Reaction (PCR) technique (Amplicor™, Roch Diagnostics, Branchburg, NJ, USA). The study was initiated in the April 2014 through 2016. All participants were assigned to the following groups:

- **Group I:** Comprised 108 chronic HBV patients with HCC
- **Group II:** Comprised 92 chronic HBV patients without HCC
- **Control group:** Comprised 85 HCC patients without HBV

Detection of VDR gene polymorphisms: Genomic DNA was prepared from EDTA-collected peripheral blood leucocytes, by using Qiagen DNA isolation kit (Wizard Genomic DNA Purification kit, Promega Corporation, Madison, WI). Genotyping was performed using a commercial PCR sequence-specific primer (SSP) (Olerup SSP; One Lambda Inc., Canoga Park, California, USA). VDR genotype was determined using PCR amplification followed by restriction fragment length polymorphisms; RFLP assay[21]. Three fragments of VDR gene were amplified; Bs fragment (745 base pair; bp), FokI fragment (265 bp) and Ap fragment (825 bp). They represent three regions of receptor gene[22]. The primer sequences of VDR polymorphisms at the four loci are shown in Table 1.

The PCR mix contained 5 μL of each primer, 5 μL buffer, 1.5 μL $MgCl_2$ (50 mM), 5 μL template DNA (50-100 ng), 5 μL dNTPs (2 mmol L^{-1}), Taq polymerase 2 μL (MBI Fermentas, St. Leon-Rot, Germany) and H_2O 26.5 μL. A total of 40 cycles of PCR were performed in a PTC-100-60 (M.J. Research, Watertown, MA, USA). An initial denaturation of DNA template at 95°C for 2 min followed by a denaturation step for 45 sec at 94°C, an annealing step for 45 sec at optimum temperature and an extension reaction at 72°C for 1 min. The optimum temperature is 61°C for Bs fragment (BsmI), 58°C for FokI and 67°C for the Ap fragment. After the last PCR cycle, a final extension step for 2 min at 72°C was added[23,24]. The amplified products were digested with BsmI, FokI, ApaI and TaqI restriction endonucleases, electrophoresed on 2% horizontal agarose gel, stained with 0.5 mg mL^{-1} of ethidium bromide and visualized under UVB-illumination using the E-Gel Precast Agarose Electrophoresis System (Invitrogen Life Technologies, PA4 9RF Paisley, UK)[22,25]. The presence of BsmI, FokI, ApaI or TaqI restriction sites was defined by the lower-case 'b', 'f', 'a' or 't', respectively. Their absence was defined by the upper-case 'B', 'F', 'A' or 'T', respectively. Digestion with BsmI restriction endonuclease resulted in three fragments (825, 650 and 175 bp) if the restriction site was present and in two fragments (650 and 175 bp) if the restriction site was absent. Digestion with FokI restriction endonuclease resulted in two fragments (196 and 69 bp). Digestion with ApaI restriction endonuclease produced two fragments (531 and 214 bp) if the site of restriction was present. TaqI restriction resulted in three fragments (205, 290 and 245 bp) when TaqI polymorphic site is present and in two fragments (245 and 495 bp) when TaqI polymorphic site is absent[26]. To improve validity and quality of genotyping, re-genotyping of 20% of samples were done by other laboratory personnel and there were no discrepancies in genotyping. Also, confirmation of genotyping of 10% randomly selected samples was done by DNA sequencing.

Statistical analysis: Data were analyzed using SPSS software (Version 17.0). Quantitative data were expressed as (Mean±SD) while qualitative data and categorical variables were expressed as number and percentage. Correlations were evaluated using the Spearman rank correlation coefficient test. Kruskal-Wallis One-Way Analysis Of Variance (ANOVA) compares more than two groups. Subgroups (percentages of patients) were compared by using the McNemar test. Categorical variables were compared using the chi-square (χ^2) test[27]. Hardy-Weinberg equilibrium was used to assess the genotype frequency of each SNP. This H-W equilibrium was then assessed using chi-square (χ^2) test. Variables that achieved statistical significance ($p \leq 0.5$) with the univariate analysis were included in multiple logistic regression analysis with forward stepwise (likelihood ratio) to evaluate the independent factors associated with risk of HCC development. Also, the Odds Ratios (ORs) with 95% Confidence Intervals (CIs) of the association between HCC and genotypic frequencies were estimated using multiple logistic regression analysis after

controlling for other covariates e.g., age, gender. Allele frequency was calculated as the number of occurrences of the test allele in the population divided by the total number of alleles. Carriage rate was calculated as the number of individuals carrying at least one copy of test allele divided by the total number of individuals. For all statistical studies, $p<0.05$ was considered to be statistically significant.

RESULTS

Primer sequences of VDR polymorphisms at four loci of VDR gene were shown in Table 1. Base change was G>A for BsmI, C>T for FokI, G>T for ApaI and G>T for TaqI. The Bs fragment contains the BsmI restriction site with one primer in exon 7 and the other in intron 8. FokI fragment contains the FokI restriction site with the primer in exon 2. The Ap fragment contains the ApaI and TaqI restriction sites with one primer in intron 8 and the other in exon 9.

There were no significant differences as regard gender, age between the HCC and non HCC groups ($p>0.05$). Also, non significant differences were detected as regard HBV infection time, family history of cancer and serum25OH-vitamin D3 levels between HCC and non HCC groups ($p>0.05$). Serum alpha fetoprotein levels were significantly higher in HCC than non HCC subjects ($p<0.001$). There was a highly significant difference in BMI between HCC and non HCC patients ($p<0.01$). Liver cirrhosis frequency was statistically and significantly higher in patients with HCC than those without HCC ($p<0.001$) (Table 2).

It is observed that there were no significant differences as regard age, gender, HBV infection time, family history of cancer and serum 25OH-vitamin D3 levels (ANOVA>0.05). Also, there were no statistically significant differences as regard serum alpha fetoprotein levels, frequency of liver cirrhosis and BMI (ANOVA$\leq$0.001). In HCC group, although there was a statistically significant higher frequency ($p = 0.011$) of liver cirrhosis in patients with HBV infection than patients without HBV infection, there were no significant differences as regard AFP and BMI ($p>0.05$) (Table 3).

Genotypic frequencies in both HCC and non-HCC patients infected with CHB are shown in Table 4. All SNPs genotypic frequencies were in Hard-Weinberg equilibrium in control subjects (all $p>0.05$). Among patients with chronic HBV infection, there were statistically significant differences

Table 1: Primer sequences of single nucleotide polymorphisms of VDR gene at the four loci

Single nucleotide polymorphisms (SNP)	Parameters
BsmI; 745 bp, rs1544410	
Forward primer	5′-CAACCAAGACTCAAGTACCGCGTCAGTGA-3′ (F)
Reverse primer	5′-AACCAGCGGAAGAGGTCAAGGG-3′ (R)
Exon	7
Intron	8
Base change	G>A
FokI; 265 bp, rs2228570	
Forward primer	5′-AGCTGGCCCTGGCACTGACTCTGCTCT-3′(F)
Reverse primer	5′-ATGGAAACACCTTGCTTCTTCTCCCTC-3′ (R)
Annealing temp.	58 °C
Exon	2
Base change	C>T
ApaI and TaqI; 825 bp	
Forward primer	5′-CAGAGCATGGACAGGGAGCAA-3' (F)
Reverse primer	5′-GCAACTCCTCATGGCTGAGGTCTC-3′ (R)
Exon (TaqI)	9, codon 352
Intron (ApaI)	8
Base change	G>T in ApaI; and T>C in TaqI
rs no	ApaI (rs7975232), TaqI (rs731236)

Table 2: Basic, demographic and clinical characteristics of patients with and without HCC

Parameters	HCC group	Non HCC group	p-value
Number	193	92	-
Age (M±SD)	52.6±6.9	50.1±7.3	0.361
Gender (F/M)	98/95	45/47	0.254
Family cancer history (yes/no)	52/141	19/73	0.182
HBV infection time (months)	15.8±4.1	13.9±3.9	0.053
Serum alpha fetoprotein level (ng mL^{-1})	2461±187	14±2.1	0.001
25OH -Vitamin D3 (ng mL^{-1})	22.1±6.14	23.15±8.28	0.066
Body mass index (kg m^{-2})	19.9±3.1	25.6±1.9	0.007
Liver cirrhosis (yes/no)	140/53	53/39	0.001

M±SD: Mean±Standard Deviation

Table 3: Basic, demographic and clinical characteristics of patients of the three groups

	HCC group		Non HCC	
Parameters	Control group (HCC without HBV)	Group I (HCC with HBV)	Group II (HBV without HCC)	ANOVA
Number	85	108	92	
Age (M±SD) years	53.2±7.2	52.3±5.6 2.3±5.9	50.1±7.3	0.253
Gender (Female/Male)	41/44	57/51	45/47	0.342
Family cancer history (yes/no)	23/62	29/79	19/73	0.082
HBV infection time (months)	No HBV infection	15.8±4.1	13.9±3.9	0.062
Body mass index (kg m^{-2})	20.3±2.4	20.1±1.7	25.6±1.9	0.031
Serum alpha fetoprotein level (ng mL^{-1})	2368±121	3632±171	14±2.1	0.003
25OH -Vitamin D3 (ng mL^{-1})	24.2±4.14	23.52±7.3	23.9±3.3	0.131
Liver cirrhosis (yes/no)	40/45	100/8	53/39	0.011

M±SD: Mean±Standard Deviation

Table 4: Genotypic frequencies of the VDR gene in both HCC and non HCC patients with CHB

		HCC (108)		Non HCC (92)			
SNPs	Haplotypes	No.	%	No.	%	Adjusted OR (95% CI)	Adjusted p-value
BsmI	GG	29	26.85	24	26.09	1	
	GA	52	48.15	46	50.00	0.936 (0.656-1.317)	0.687
	AA	27	25.00	22	23.91	1.016 (0.706-1.457)	0.835
	G	110	50.93	94	51.09	1	
	A	106	49.07	90	48.91	1.006 (0.839-1.205)	0.842
FokI	CC	27	25.00	33	35.87	1	
	CT	48	44.44	41	44.56	1.431 (1.051-1.994)	0.0018
	TT	33	30.56	18	19.57	2.241 (1.569-3.195)	0.007
	C	102	47.22	107	58.15	1	
	T	114	52.78	77	41.85	1.553 (1.297-1.861)	0.001
ApaI	GG	28	25.93	23	25.00	1	
	GT	53	49.07	45	48.91	0.967 (0.701-1.235)	0.799
	TT	27	25.00	24	26.09	0.924 (0.698-1.452)	0.201
	G	109	50.46	91	49.46	1	
	T	107	49.54	93	50.54	0.961(0.775-1.184)	0.173
TaqI	TT	26	24.07	21	22.83	1	
	TC	54	50.00	44	47.83	0.991 (0.721-1.365)	0.979
	CC	28	25.93	27	29.34	0.838 (0.647-1.404)	0.602
	T	106	49.07	86	46.74	1	
	C	107	50.93	98	53.26	0.886 (0.702-1.105)	0.573

CI: Confidence interval, SNPs: Single nucleotide polymorphisms, HCC: Hepatocellular carcinoma, CHB: Chronic hepatitis B

between HCC and non HCC groups regarding the genotype frequencies of VDR FokI C>T polymorphism. In HCC patients there was a higher prevalence of FokI TT genotype than non HCC patients (30.56% versus 19.57%, p = 0.007). Moreover, HCC patients had a higher T allele frequency of FokI than non HCC patients (52.78% versus 41.85%, p<0.001). Multivariate regression analyses were done to identify the independent risk factor for the development of HCC. In VDR FokI C>T polymorphism and using FokI CC as a reference, the data showed that TT carriage had a statistically significantly higher risk for HCC development after adjusting other covariates including age, gender, HBV infection time, family cancer history, BMI, serum 25OH-Vitamin D3 levels and serum AFP levels [adjusted OR (95% CI) = 2.241 (1.569-3.195), p = 0.007]. Also, with C allele as a reference, T allele carriage had a significantly higher risk for development of HCC after adjustments of all previous covariates [adjusted OR 95% CI = 1.553 (1.297-1.861), p<0.001]. The genotype and allele frequencies of other SNPs of VDR gene at BsmI, ApaI and TaqI loci had no significant differences between HCC and non-HCC patients (all p>0.05). Also, in this study, Multivariate regression analyses showed no association between other SNPs (BsmI, ApaI and TaqI) of VDR gene and risk of HCC development.

Genotypic frequencies of VDR gene in HCC patients with chronic HBV infection (108) and HCC patients without chronic HBV infection (85) are demonstrated in Table 5. The data showed that VDR genotype frequencies were similar between HCC with HBV infection and HCC without HBV infection. All SNPs of VDR gene at BsmI, FokI, ApaI and TaqI loci showed no significant difference between HCC with HBV infection and HCC without HBV infection (all p>0.05). Also, multivariate

Table 5: Genotypic frequencies of VDR gene in HCC patients with or without CHB

	HCC with CHB (108)		HCC without CHB (85)			
SNPs	No.	%	No.	%	Adjusted OR (95%CI)	Adjusted p-value
BsmI						
GG	29	26.85	23	27.06	1	
GA	52	48.15	41	48.23	1.006 (0.645-1.562)	0.976
AA	27	25.00	21	24.71	1.02 (0.598-1.989)	0.384
G	110	50.93	87	51.18	1	
A	106	49.07	83	48.82	1.01 (0.742-1.323)	0.471
FokI						
CC	27	25.00	20	23.53	1	
CT	48	44.44	40	47.06	0.889 (0.597-1.364)	0.696
TT	33	30.56	25	29.41	0.978 (0.618-1.511)	0.702
C	102	47.22	80	47.06	1	
T	114	52.78	90	52.94	0.993 (0.701-1.281)	0.895
ApaI						
GG	28	25.93	15	17.65	1	
GT	53	49.07	48	56.47	0.592 (0.453-0.979)	0.058
TT	27	25.00	22	25.88	0.657 (0.413-1.181)	0.359
G	109	50.46	78	45.88	1	
T	107	49.54	92	54.12	0.832 (0.519-1.415)	0.229
TaqI						
TT	26	24.07	21	24.71	1	
TC	54	50.00	45	52.94	0.969 (0.616-1.391)	0.541
CC	28	25.93	19	22.35	1.19 (0.879-1.695)	0.919
T	106	49.07	87	51.18	1	
C	107	50.93	83	48.82	1.058 (0.785-1.395)	0.897

CI: Confidence interval, SNPs: Single nucleotide polymorphisms, HCC: Hepatocellular carcinoma, CHB: Chronic hepatitis B

Table 6: Association between FokI C>T polymorphism and clinicopathological features in HCC patients with CHB

FokI	C>T	No.	%	No.	%	Adjusted OR (95% CI)	Adjusted p-value
Tumor size		>30 mm (55)		<30 mm (53)			
FokI	CC	13	23.64	14	26.41	1	
	CT	27	49.09	26	49.06	1.112(0.601-1.53)	0.455
	TT	15	27.27	13	24.53	1.243(0.598-1.998)	0.065
Cancer stage		III, IV (56)		I, II (52)			
FokI	CC	10	17.86	15	28.85	1	
	CT	27	48.21	24	46.15	1.688(1.005-2.631)	0.056
	TT	19	33.93	13	25.00	2.192(2.294-3.534)	0.001
Liver cirrhosis history		Presence (53)		Absence (55)			
FokI	CC	10	18.87	16	29.09	1	
	CT	23	43.40	26	47.27	1.415(0.984-1.989)	0.131
	TT	20	37.73	13	23.64	2.462(1.651-4.012)	0.001
Lymph node metastasis		Yes (52)		No (56)			
FokI	CC	11	21.15	17	30.36	1	
	CT	23	44.23	26	46.43	1.259(0.894-2.192)	0.037
	TT	18	34.62	13	23.21	1.877(1.195-3.124)	0.003
Portal invasion		Yes (51)		No (57)			
FokI	CC	13	25.49	15	26.32	1	
	CT	27	52.94	26	45.61	1.198(0.896-1.999)	0.298
	TT	11	22.57	16	28.07	0.793(0.504-1.387)	0.529

CI: Confidence interval, SNPs: Single nucleotide polymorphisms, HCC: Hepatocellular carcinoma, CHB: Chronic hepatitis B

regression analyses showed no association between SNPs of VDR gene at BsmI, FokI, ApaI and TaqI loci and the presence or absence of HBV infection in HCC patients.

The association between VDR gene polymorphisms and the clinicopathological features in HCC Egyptian patients infected with chronic HBV infection were further analyzed in Table 6. It was observed that all SNPs at BsmI, TaqI and ApaI loci had no statistical significance when all HCC patients were stratified by tumor stage, tumor size, lymph node metastasis, presence of liver cirrhosis history and portal invasion (all $p>0.05$, data not shown). On the other hand, we found that the SNPs at FokI locus were statistically significantly different when all HCC patients were stratified by tumor stage, lymph node metastasis and the presence of liver cirrhosis history ($p<0.05$) but not portal invasion and tumor size ($p>0.05$). Multivariate logistic regression analyses were further

Table 7: Association between FokI C>T polymorphism and laboratory features in HCC patients with CHB

HCC patients with CHB	Serum AFP* (ng mL^{-1})	Serum ALT* (μL^{-1})	Serum AST* (μL^{-1})
FokI			
CC	2124±58.5	59±3.5	70±4.2
CT	2370±75.8	68±4.1	78±5.6
TT	3614±87.8	112±9.2	132±11.3
P^1 value	0.001	0.001	0.001
P^2 value	0.004	0.008	0.009

AFP: Alpha fetoprotein, ASTL Aspartate transaminase, ALT: Alanine transaminase levels, *Mean±Standard Deviation, CHB: chronic hepatitis B, P^1: TT versus CC, P^2: TT versus CT

performed to examine the role of SNPs at FokI locus in determination of the clinicopathological features in HCC patients. When CC genotype was taken as a reference, the TT genotype carriage of FokI polymorphisms was found to be associated with presence of liver cirrhosis history (adjusted OR = 2.462, p<0.001), lymph node metastasis (adjusted OR = 1.877, p = 0.003) and advanced cancer stage (adjusted OR = 2.192, p = 0.001). On the other hand, there were no associations between the FokI polymorphisms, tumor size and portal invasion (p>0.05). The SNP at other loci including BsmI, ApaI and TaqI did not show significant association with the clinicopathological features of HCC (all p>0.05, data not shown).

The HCC patients with CHB carrying FokI TT genotype had much higher and statistically significant serum AFP, ALT and AST levels than patients carrying either CT or CC genotype (Table 7, all p<0.01). On the other hand, serum AFP, ALT and AST levels were similar among genotype carriers of BsmI, ApaI and TaqI. Moreover, there were no differences of serum AFP, ALT and AST levels among genotype carriers of BsmI, FokI, ApaI and TaqI in non-HCC patients infected with CHB (all p>0.05, data not shown).

DISCUSSION

In this study, the possible association between VDR Gene polymorphisms and HCC susceptibility in Egyptian populations infected with CHB was investigated. It is observed that only SNP of VDR gene at FokI locus (C>T) were associated with increased susceptibility of HCC development. Moreover, the relation between SNPs of VDR gene and clinico-pathological characteristics in HCC Egyptian patients infected with chronic HBV was further analyzed. It is found that the SNPs at FokI locus were statistically significantly (p<0.05) different after stratification of all HCC patients by cancer stage, lymph node metastasis and liver cirrhosis, but not by portal vein invasion and tumor size. Using multivariate logistic regression analyses, we further investigated the role of SNPs at FokI locus in determination of the clinico-pathological characteristics in HCC patients. We observed that the carriage of FokI TT genotype was associated with presence of liver cirrhosis history, metastasis of lymph node and advanced cancer stage. Since there is scarcity in studies investigating this aspect, our data may provide new information supporting this issue.

The HCC is one of the most complex, multistep and multifactorial processes with multiple risk factors[12]. Hepatic carcinogenesis is mediated by a variety of genetic factors[8]. However, the association of vitamin D and HCC is still inconclusive. Viral hepatitis infections are associated with increased oxidative stress in hepatocytes resulting in DNA changes, instability and genetic polymorphisms of various genes which may provide an increased risk of liver cirrhosis and/or HCC development[28,29]. It was previously evidenced that the polymorphisms of some specific genes may be predictive for the occurrence of HCC in patients with viral hepatitis and alcoholic liver cirrhosis[30,31].

The VDR is an intracellular hormone receptor which specifically binds 1, 25-dihydroxyvitamin D (the biologically active form of vitamin D) then interacts with specific nucleotide sequences (response elements) of certain target genes to produce a variety of genomic effects[32].

It had been demonstrated that the VDR gene is located on chromosome 12q12-q14. Also, it has been reported that several Single Nucleotide Polymorphisms (SNPs) in the VDR gene are associated with tumorigenesis in various organs but these observations are conflicting[13,33].

In recent years, several polymorphisms have been described in the VDR genes at several loci, such as BsmI, FokI, TaqI and ApaI. These VDR gene polymorphisms identified by allelic variation in restriction enzyme sites are able to alter the activity of VDR proteins[34].

The FokI C>T polymorphism detected by Pcr-restriction Fragment Length Polymorphism (PCR-RFLP) is located in Exon 2, the coding region of the VDR gene resulting in the production of an altered and functionally less effective VDR protein (three amino acids longer)[35]. Although the genetic background still remains unclear, it has been demonstrated that VDR polymorphism at FokI C>T has an essential role in VDR gene transcriptional activation. Compared with the FokI T/T genotype, FokI C/C genotype had 1.7 fold greater function of the transcriptional activation of a reporter construct which is vitamin D-dependent and controlled by vitamin D response element in transfected HeLa cells. These data hypothesized that decreased activity of VDR could be associated with increased susceptibility to cancer risk and more aggressive disease[33].

Previous studies demonstrated that the F allele (F/f) of FokI C>T polymorphism of VDR gene was genetically linked to autoimmune hepatic disease especially Primary Biliary Cirrhosis (PBC) in German populations[36]. In a Chinese study, autoimmune hepatitis was linked to FokI C>T (F/f) polymorphism and BsmI G>A polymorphism was associated with primary biliary cirrhosis[37]. In an Italian study, GG genotype carriage of BsmI G>A polymorphism and TT genotype carriage of TaqI T C polymorphism were associated with higher HCC occurrence rate among patients underwent liver transplantation due to liver cirrhosis caused by HCV, HBV or alcoholic liver disease. Moreover, this association was more specific in patients with alcoholic liver disease[15].

Another study revealed that ApaI polymorphism of VDR gene plays a crucial role in identifying those at risk of HCC development among chronic hepatitis C patients[38].

The present study also showed that only the SNPs of VDR gene at FokI locus (CT) were significantly associated with increased susceptibility to HCC development in HCC Egyptian patients with CHB ($p<0.05$). On the other hand, other SNPs of VDR gene at TaqI, BsmI and ApaI loci were not associated with or related to HCC development. Patients with HCC had statistically significantly higher frequency rate of liver cirrhosis ($p<0.05$) than patients without HCC. These findings are in accordance to those obtained by Yao *et al.*[39] who revealed that FokI polymorphisms could be used as a molecular marker to predict the risk of HCC development and severity in patients infected with HBV.

This study could suggest that FokI (C>T) polymorphism of VDR gene enhance the occurrence of liver cirrhosis and may relate to HCC development. The discrepancies in the SNPs of VDR gene among these studies could be related to the ethnic differences.

The SNP of VDR gene at FokI C>T (F/f) locus had been reported to have a prognostic role in cancer patients. In a study by Hama *et al.*[40], the TT genotype of FokI C>T polymorphism was associated with poor prognosis and advanced disease stage in patients with squamous cell carcinoma of head and neck. While, other SNP of VDR gene at BsmI, ApaI and TaqI loci were not significantly associated with progression-free survival ($p>0.05$).

The prognostic analyses were not done in this study; however, we analyzed the relation between SNPs of VDR gene and the clinicopathological characteristics in HCC Egyptian patients with chronic HBV infection using multivariate logistic regression analyses, we further investigated the role of SNPs at FokI locus in the determination of the clinicopathological characteristics in HCC patients. We observed that the carriage of TT genotype of FokI polymorphisms was associated with the presence of liver cirrhosis history, metastasis of lymph node and advanced cancer stage. Other SNPs at BsmI, TaqI and ApaI loci had no statistically significant role in the determination of the clinicopathological characteristics in the HCC patients.

In addition, FokI TT genotype carriers had much higher and statistically significant ($p<0.05$) serum AFP, ALT and AST level than patients carrying either CT or CC genotypes. On the other hand, serum AFP, ALT and AST levels were similar among genotype carriers at BsmI, ApaI and TaqI. Moreover, there were no differences of serum AFP, ALT and AST levels among BsmI, FokI, ApaI and TaqI genotype carriers in non-HCC patients infected with HBV.

The conventional factors associated with HCC prognosis are cancer stage, differentiation, lymph node metastasis and the presence of cirrhosis. Hence, our data could imply that the carriage of TT genotype of FokI polymorphisms had a much poorer prognosis than CT and CC genotype carriage.

The limitations of this study were that enrollment bias as it is a case-control hospital-based study and enrollment only HBV-infected subjects. So, further studies with larger sample size with enrollment of HCV-infected patients are warranted.

CONCLUSION

The present study speculated that only SNPs FokI (C>T) of VDR gene were associated with increased susceptibility to HCC development and have a determinant role in clinicopathological features in of HCC Egyptian patients with chronic HBV infection.

SIGNIFICANCE STATEMENT

The VDR gene polymorphism at FokI locus was associated with increased HCC susceptibility. So, it could be used as a molecular marker predicting the risk and evaluating the severity of HCC in Egyptian patients infected with CHB.

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