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Impact of FokI (rs10735810) and BsmI (rs1544410) on Treatment of Chronic HCV Patients With Genotype 4

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Background and Aim: Chronic infection with hepatitis C virus (HCV) is a huge problem both globally and at the level of the individual patient. Our aim is to detect the influence of vitamin D receptor gene polymorphisms (BsmI and Fok1) and vitamin D level in HCV patients under treatment with interferon. *Subject and Methods:* Blood samples were taken from 103 HCV patients all of them are genotype 4. They were divided into responders ($n = 63$) and nonresponders ($n = 40$) according to their response to interferon treatment. Also 120 subjects with matched age and sex were enrolled as controls. All subjects were subjected to history taking, general examination, liver function tests, hepatitis markers, HCV quantitation by real-time polymerase chain reaction (PCR), DNA extraction from whole blood, PCR-restriction fragment length polymorphism (RFLP) for genotyping, and quantitation of vitamin D level by ELISA. *Results:* There were significant differences between responders and nonresponders in the mean values of vitamin D ($P = 0.001$) as well as the prevalence of single nucleotide polymorphism (SNP) BsmI (Bb) ($P = 0.02$). Meanwhile, no significant differences in Fok1 genotype between responders and nonresponders to interferon therapy of HCV patients in all genotypes [FF, Ff, ff) ($P = 0.34$, 0.091, and 0.43), respectively. *Conclusion:* BsmI and vitamin D level in chronic liver disease patients are predictors of response to combination therapy of HCV. J. Clin. Lab. Anal. **30**:1021–1027, 2016. © 2016 Wiley Periodicals, Inc.

Key words: hepatitis C; vitamin D; polymorphism

INTRODUCTION

Around 170 million people worldwide have chronic hepatitis C (CHC) causing a substantial burden of chronic liver disease globally (1). Vitamin D deficiency is more prevalent in CHC subjects than healthy controls, even in those with minimal liver fibrosis. The majority of subjects with CHC are vitamin D deficient (<50 nmol/l) with 25% having severe deficiency (<25 nmol/l) (2).

Vitamin D is a potent immunomodulator that favors innate immunity and cell differentiation (3). Its deficiency is very common (92%) among patients with chronic liver disease.

Variation in the allele frequency of the BsmI polymorphism is associated with primary biliary cirrhosis (4), while variation of the FokI polymorphism is associated with autoimmune hepatitis (5).Patients with vitamin D deficiency have a higher grade of hepatic necroinflammation (6), more advanced fibrosis stage (7), and may possibly have more rapid fibrosis progression (8).

The reasons why vitamin D deficiency occurs in patients with CHC are far less clear. An explanation of this finding likely requires taking into account the multiple interconnections between vitamin D, the immune response, and inflammatory status (9, 10). *Bitetto* et al. (6) showed that vitamin D supplementation improves response to antiviral treatment for recurrent hepatitis C in liver transplant recipients.

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SUBJECTS AND METHODS

This study was conducted on 103 hepatitis C virus (HCV) patients; all of them are genotype 4 and 120 healthy individuals as controls not suffering from any viral infection. Patients were selected from El fatemeya Hospital, Cairo, as naive patients not treated before; both males and females were included. Patients were treated with pegylated interferon (PEG-IFN) alpha 2b 1.5 μg/kg weekly and ribavirin (13–15 mg/kg) daily for 24 weeks and followed for 72 weeks. According to response to treatment, patients were classified into two groups: responders, who had clearance of the virus denoted by negative HCV-RNA by real-time PCR after 6 months at the end of treatment; and nonresponders, who failed to clear the virus and give positive HCV-RNA by real-time PCR.

Under complete septic condition, 5 ml venous blood samples were withdrawn and divided into two tubes from patients and controls after obtaining their written consent as follows: 3 ml blood were centrifuged at 2,000 × *g* for 5 min. The serum was then separated for determination of all serological tests including ELISA as well as HCV-RNA quantitation. Two milliliters EDTA blood was stored at −80°C to be used for molecular biology techniques.

Methods

Laboratory tests

Routine tests

Complete blood picture using (SWELAB ALFA CELL COUNTER) liver biochemical profile: serum bilirubin (total and direct) (using DAIOMOD diagnostic kits), transaminases (ALT and AST), alkaline phosphatase, total protein, serum albumin (all by using Reactivos GPL), and prothrombin time and concentration (using BIOMED diagnostic) were done to all samples.

CHC-infected patients were subjected to abdominal ultrasound and liver biopsy was done for all patients to determine the grades of activity and fibrosis according to Metavir Scoring System. All biopsies were classified according to Metavir Scoring System (11) into five stages of fibrosis (F0: no fibrosis; F1/2: enlargement of portal tract without or with rare septa formations; F3/4: numerous septa without or with cirrhosis) and four grades (inflammations) of histological activity (A0/1: none or mild; A2/3: moderate or severe) based on the intensity of necroinflammatory lesions.

Serological tests for chronic hepatitis markers

Anti-HCV EIA and Anti-HBc EIA were done using (Abbott Laboratories, Ludwigshafen, Germany).

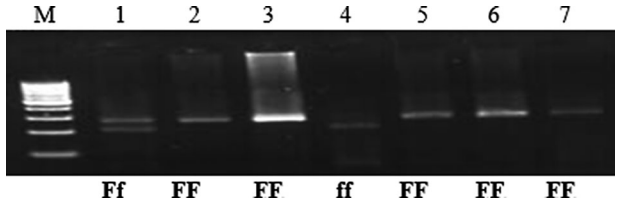

Fig. 1. Agarose gel electrophoresis (3.5%) of PCR-RFLP technique of amplified FOKI genotype.

HCV RNA by real-time PCR technique

Qiaplex thermal cycler was used (Applied Biosystems) for all samples to diagnose HCV infection and viral load.

Quantitation of serum level of 25(OH) vitamin D

Serum was used for quantitation of 25(OH) vitamin D by ELISA (Immun Diagnostik, Germany). The assay utilizes a competitive ELISA technique with a selected monoclonal antibody recognizing 25(OH) vitamin D.

Molecular biology tests

DNA was extracted from whole blood using DNA extraction kit (Qia-amplification extraction kit, Qiagen). The FOKI polymorphism in exon 2 and BSMI polymorphism in intron 8 were determined by using these primers: forward: 5′-AGCTGG CCC TGG CAC TGA CTA TGC TCT-3′, reverse: 5′-ATG GAA ACA CCT TGC TTC TTC TCC CTC-3′ for the FOKI and 5′-CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA-3′, reverse: 5′-AAC CAG CGC GAA GAG GTC AAG GG-3′ for BSMI (metabion-inter-national AG, lena-christ-str. 44, 82152 martinsried, Germany).

Genotyping of FokI C > T (rs10735810): DNA was denatured for 5 min at 94°C, the reaction mixture was subjected to 35 cycles of denaturation for 30 s at 94°C, 30 s annealing at 61°C, and 1 min extension at 72°C. The 265 bp PCR product was digested with 8 U FOKI (BSeGI) restriction endonuclease overnight at 37°C. This enzyme was supplied by Ferments international Inc. The digested products were separated on 3.5% agarose gel with ethidium bromide staining and ultraviolet transillumination. The FF genotype (homozygote of common allele) lacked a FOKI restriction site and showed only one band of 265 bp. The ff genotype (homozygote of infrequent allele) generated two bands of 196 and 69 bp. The heterozygote displayed three fragments of 265, 196, and 69 bp, designated as Ff (Fig. 1).

Genotyping of BsmI A > G (rs1544410): The PCR cycling condition was: 94°C for 5 min for denaturation followed by 35 cycles at 94°C for 1 min, 64°C for 1 min, and 72°C for 90 s and a terminal extension at 72°C for 10 min. After amplification the PCR product 850 bp was

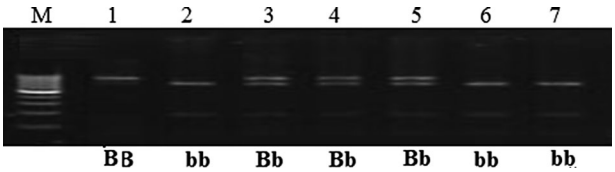

Fig. 2. Agarose gel electrophoresis (4%) of PCR-RFLP technique of amplified BsmI genotype.

digested with restriction endonuclease BSMI enzyme after incubation at 37°C overnight and electrophoresed in a 4% agarose gel containing ethidium bromide and then visualized by UV transilluminator. Subjects homozygote for the BSMI restriction site are designated bb and show two fragments at 650 and 200 bp, while homozygous for the absence of the site are designated BB and give one band at 850 bp and the heterozygote type gives three bands (Fig. 2).

Statistical Analyses

Data were coded and entered using the statistical package SPSS version 15. Data were summarized using mean, standard deviation, and range for quantitative variables. Comparison between two groups was done using independent sample *t*-test or ANOVA (analysis of variants) for comparing more than two groups. Correlations were done to show the relation between quantitative variables using Pearson's coefficient. *P*-values < 0.05 were considered to be statistically significant. All populations in the present study were in Hardy–Weinberg equilibrium.

RESULTS

There was a highly statistically significant difference between HCV patients compared to control subjects as regards the mean values ± SD of ALT, AST, D. bilirubin, ALB, ALK, and vitamin D ($P < 0.001$ each). Meanwhile, there was no statistically significant difference as regards the age, sex, T. bilirubin, and Alpha-Fetoprotein (AFP) (Table 1).

The present study shows that there is significant difference comparing vitamin D level and the vitamin D receptor (VDR) polymorphisms (BsmI) in HCV patients as well as controls (BB, Bb, bb) ($P = 0.001$ each) (Table 2). Table (2) shows also significant difference in the vitamin D level and the VDR polymorphisms (FoKI) in both HCV and controls (FF, Ff, ff) ($P = 0.001$ each).

Comparing responders to nonresponders, there was a highly statistically significant difference as regards the mean values ± SD of AST ($P = 0.009$), D. bilirubin ($P = 0.001$), AFP ($P = 0.000$), vitamin D ($P = 0.001$), and fibrosis ($P = 0.001$). Meanwhile, there was no statistically significant difference as regards age, sex, ALT, T. bilirubin, ALK, ALB, as well as quantitation of HCV titer (Table 3). In the present study, there is a significant difference in the prevalence of vitamin D deficiency between responders and nonresponders HCV patients ($P = 0.003$) (Table 3).

Table 4 shows no significant statistical difference in the genotypes distribution between responders and nonresponders as regards [(BB, bb)], on the other hand there is significant statistical difference as regards [(Bb)] ($P = 0.02$). Also, there is no significant statistical differences in the distribution of wild genotype (BB) versus the mutant genotypes (Bb + bb) ($P = 0.12$). The allele frequencies between the two groups showed no significant

TABLE 1. Demographic and Biochemical Data in HCV Patients and Controls

Variables	HCV patients Mean ± SD $N = 103$	Controls Mean ± SD $N = 100$	*P*-value
Age (years)	43.21 ± 8.688	40.63 ± 8.109	0.62
Sex			
Females % within group	35(34%)	14(46.7%)	0.113
Males % within group	68(66%)	16(53.3%)	
ALT (U/L)	58.56 ± 27.9	26.83 ± 3.4	0.001*
AST (U/L)	54.03 ± 18.1	28.40 ± 4.4	0.001*
T. bilirubin (mg/dl)	0.69 ± 0.3	0.73 ± 0.2	0.546
D. bilirubin (mg/dl)	0.32 ± 0.2	0.15 ± 0.06	0.001*
ALK (U/L)	150.78 ± 52.9	38.8 ± 0.2	0.001*
ALB (g/dl)	3.15 ± 0.6	4.31 ± 7.4	0.001*
AFP (ng/ml)	6.04 ± 7.4	5.86 ± 2.0	0.892
Vitamin D (nm/l)	62.4 ± 38.6	91.75 ± 64.1	0.001*

P-values < 0.05

TABLE 2. The Relation Between Vitamin D Level and BsmI and Fok1 SNPs in HCV Patients and Controls

Group		No.	Vitamin D level (nmol/l)	Std. deviation	*P*
Patients	BB	25	105.70	48.53	
	Bb	40	75.54	21.84	0.001
	bb	38	38.92	13.63	
	Total	103	62.35	38.57	
Controls	BB	16	196.37	46.9	
	Bb	64	77.06	19.48	0.001
	bb	40	61.40	61.50	
	Total	120	91.75	64.14	
Patients	FF	38	96.91	42.84	
	Ff	36	66.1	23.72	
	ff	29	37.26	13.44	0.001
	Total	103	62.35	38.57	
Controls	FF	40	135.35	77.84	
	Ff	64	74.58	12.53	0.001
	ff	16	51.4	15.86	
	Total	100	91.75	64.14	

TABLE 3. Comparison Between Demographic and Biochemical Data in Responders and Nonresponders to Interferon Therapy of HCV Patients Before Treatment

Variables	RespondersMean ± SD$N = 63$	NonrespondersMean ± SD$N = 40$	P-value
Age (years)	43.18 ± 8.800	43.25 ± 8.628	0.970
Sex			
Females % within group	24 (38.1%)	11(27.5%)	0.269
Males % within group	39(61.9%)	29(72.5%)	
ALT (U/L)	57.03 ± 27.406	60.92 ± 28.831	0.494
AST (U/L)	50.37 ± 18.622	59.80 ± 15.696	0.009*
T. bilirubin (mg/dl)	0.64 ± 0.295	0.77 ± 0.345	0.062
D. bilirubin (mg/dl)	0.25 ± 0.183	0.43 ± 0.295	0.001*
ALK (U/L)	149.99 ± 58.031	152.02 ± 44.231	0.850
ALB (g/dl)	3.25 ± 0.441	3.00 ± 0.833	0.084
AFP (ng/ml)	3.43 ± 2.72	9.83 ± 10.07	0.000*
Vitamin D (nmol/l)	78.96 ± 41.57	54.21 ± 27.57	0.001*
<25 Count % within response	1 (1.6%)	5 (12.5%)	0.003*
25–50 Count % within response	13 (21.0%)	18 (45.0%)	
50–75 Count % within response	20 (32.3%)	8 (20.0%)	
>75 Count % within response	28 (45.2%)	9 (22.5%)	
HCV RNA (PCR)	$2.23 \times 10^6 \pm 4.968 \times 10^3$	$1.53 \times 10^6 \pm 1.979 \times 10^3$	0.405
Fibrosis			
Grades 1%within group	27(42.9%)	12(30%)	0.001*
Grades 2%within group	31(49.2%)	13(32.5%)	
Grades 3%within group	4(6.3%)1(1.6%)	15(37.5%)0(0%)	
Grades 4%within group	1(1.6%)	0(0%)	

P-values < 0.05

statistical difference [B allele (wild) vs. b allele (mutant)] ($P = 0.76$).

There is no significant statistical difference in the genotypes distribution between responders and nonresponders [(FF, Ff, ff) ($P = 0.34$, 0.091, and 0.43), respectively]. Also, there is no significant statistical differences in the distribution of wild genotype (FF) versus the mutant genotypes (Ff + ff) ($P = 0.34$). Also, the allele frequencies between the two groups showed no significant statistical difference [F allele (wild) vs. f allele (mutant)] ($P = 0.884$) (Table 5).

A stepwise multivariate logistic regression shows the factors predicting response to interferon therapy of hepatitis C patients (Table 6). Only ALT ($P = 0.040$), AST ($P = 0.009$), D. bilirubin ($P = 0.013$), ALB ($P = 0.012$), AFP ($P = 0.001$), vitamin D ($P = 0.002$), and Bsm1 ($P = 0.02$) were found to be significant predictors for response. No statistical significant difference was found in the vitamin D level as regards different degrees of liver fibrosis (Table 7). Table 8 shows that Fok1 and Bsm1 SNPs were statistically significant different in fibrosis grades in HCV patients ($P = 0.007$ and 0.041, respectively).

DISCUSSION

In the present study, we found significant difference between hepatitis C patients compared to control subjects as regards ALT, AST, D. bilirubin, ALK, ALB, and vitamin D ($P < 0.001$ each). This is consistent with previous study by Mohamadnejad et al. (12), who reported that when parenchymal liver cells are damaged, aminotransferases leak from the liver into the blood, resulting in elevated levels of these enzymes in the blood stream. Also, Lange et al. (2) showed that vitamin D deficiency is more prevalent in CHC subjects than healthy controls.

Our results revealed that there is no significant difference comparing vitamin D level and the VDR receptor polymorphisms (BsmI) in HCV patients (BB, Bb, bb) (105.70, 75.54, and 38.92 nmol/l), respectively

TABLE 4. Prevalence of BsmI SNPs in Responders and Nonresponders to Interferon Therapy of HCV Patients

	Genotypes						
VDR (BsmI A > G) B/b	BB	Bb	bb	BB	Bb, bb	B allele	b allele
Responders	19.0%	47.6%	33.4%	19.0%	81.0%	48.2	51.8
Nonresponders	32.5%	25%.0	42.5%	32.5%	67.5%	54.0	55.0
P-value	0.12	0.02	0.35	0.12		0.76	

TABLE 5. Prevalence of Fok1 SNP in Responders and Nonresponders to Interferon Therapy of HCV Patients

	Genotypes						
VDR (Fok1 C > T) F/f	FF	Ff	ff	FF	Ff, ff	F alleles	f alleles
Responders	33.3%	41.3%	25.4%	33.3%	66.7%	54.0	46.0
Nonresponders	42.5%	25.0%	32.5%	42.5%	57.5%	55.0	54.0
P-value	0. 34	0.091	0.43	0.34		0.884	

TABLE 6. A Stepwise Multivariate Logistic Regression Showing the Factors Predicting Response to Interferon Therapy of HCV Patients

Variables	*P*-value	Odd ratio	Odds ratio (95%CI)
ALT (U/L)	0.040*	0.957	(0.917–0.998)
AST (U/L)	0.009*	1.114	(1.027–1.208)
D. bilirubin (mg/dl)	0.013*	4.1×10^3	$(5.9–2.9 \times 10^6)$
ALB (g/dl)	0.012*	0.099	(0.016–0.605)
AFP (ng/ml)	0.001*	2.419	(1.426–4.103)
Vitamin D (nmol/l)	0.002*	0.873	(0.801–0.951)
Bsm1 polymorphism	0.02*	3.02	(1.65–5.51)

P-values < 0.05

($P = 0.001$). Also, there is no significant difference comparing vitamin D level and BsmI in the healthy control group (BB, Bb, bb) (196.37, 77.06, 61.40 nmol/l), respectively ($P = 0.001$). The lowest level of vitamin D was found in the mutant genotype bb (38.92 and 61.40 nmol/l) in HCV and controls, respectively. These results coincide with that of Schuch et al. (13) who stated that (bb) homozygous for the BsmI VDR variant was associated with lower 25(OH) D3 values than other genotypes.

Our study revealed that there is no significant difference in the allele and genotype frequencies of the VDR polymorphisms (Bsm1 and Fok1) between the patients and controls. The data agreed with report in the literature on VDR polymorphisms in patients with chronic liver disease (14).

Our results revealed that there is a significant difference in the prevalence of vitamin D deficiency between responders and nonresponders to interferon therapy of CHC patients ($P = 0.003$). These results are consistent with reports dealing with the association between vitamin D status and outcome of antiviral therapy for chronic HCV viral infection. Petta et al. (15) have retrospectively analyzed a cohort of 167 patients treated with peg-interferon and ribavirin for hepatitis C, and detected an association between lower vitamin D serum levels and failure to achieve sustained virological response (SVR). Also, Gutierrez et al. (16) have shown that vitamin D3 increases VDR protein expression and inhibits viral replication in cell culture.

As regards to fibrosis in our patients, Larrubia et al. (17) confirmed that persistent HCV infection modulates the balance between immunostimulatory and inhibitory cytokines that can prolong inflammation and lead to fibrosis and chronic liver diseases.

In the present study we failed to find any relation between vitamin D level and the stages of fibrosis. Meanwhile, Petta et al. (18) showed that a low serum vitamin D level was related to severe fibrosis and low responsiveness to interferon-based therapy in genotype 1 CHC. It was shown that adding vitamin D to conventional Peg/RBV therapy for naïve, genotype 1 patients with chronic HCV infection significantly improves SVR (19).

Lange et al. (2) showed that vitamin D deficiency is more prevalent in CHC subjects than healthy controls, even in those with minimal liver fibrosis. The majority of subjects with CHC are vitamin D deficient (<50 nmol/l) with 25% having severe deficiency (<25 nmol/l).

Our study also revealed that there is a significant difference in fibrosis grades ($P = 0.001$) between responders (91.9% with grade 1, 2 and 8.1% with grade 3, 4) and nonresponders (62.5% with grade 1, 2 and 37.5% with 3, 4 grades) groups. This is consistent with previous study by Everson et al. (20) who reported that advanced hepatic fibrosis is a negative predictor of SVR to therapy.

TABLE 7. The Vitamin D Levels in Different Fibrosis Grades in HCV Patients' Responders and Nonresponders

Fibrosis grade		*N* (40)	Vitamin D (nmol/L)	Std. deviation	Minimum	Maximum	*P*-value
Nonresponder	1	12	55.515	17.8367	34.5	86.5	0.146
	2	13	41.479	16.6081	17.0	73.4	
	3	15	52.690	20.9331	20.4	91.6	
Responder	1	27	73.144	24.2178	37.7	134.5	0.435
	2	31	84.180	39.7928	32.8	250.0	
	3 + 4	5	83.422	26.4851	49.9	112.7	

TABLE 8. FOK1 and BSM1 SNPs in Different Fibrosis Grades in all HCV Patients

			Fibrosis				
			1	2	3 and 4	Chi-square	*P*
VDR (Fok1 C > T) F/f	FF	Count	9	18	11	14.03	0.007
		% within fibrosis	23.1%	40.9%	55.0%		
	Ff	Count	22	11	3		
		% within fibrosis	56.4%	25.0%	15.0%		
	ff	Count	8	15	6		
		% within fibrosis	20.5%	34.1%	30.0%		
VDR (BsmI A > G) B/b	BB	Count	8	9	8	9.95	0.041
		% within fibrosis	20.5%	20.5%	40.0%		
	Bb	Count	21	16	3		
		% within fibrosis	53.8%	36.4%	15.0%		
	bb	Count	10	19	9		
		% within fibrosis	25.6%	43.2%	45.0%		

Another researcher, Poynard et al. (21) found that patients with established cirrhosis are more resistant to IFN-α therapy than those who have fibrosis, whereas patients with fibrosis are less responsive to IFN-α therapy than those without fibrosis. This finding may be explained by the fact that changes in intrahepatic inflammatory response and mediators during fibrosis progression may affect combined PEG-IFN and ribavirin response (22).

Multivariate logistic regression analysis identified the following predictors of response: ALT (OR, 0.957; 95%CI, 0.917–0.998; $P = 0.040$), AST (OR, 1.114; 95%CI, 1.027–1.208; $P = 0.009$), D. bilirubin (OR, 4.1×10^3; 95%CI, 5.9–2.9×10^6; $P = 0.015$), ALK (OR, 1.02; 95%CI, 1.000–1.032; $P = 0.057$), ALB (OR, 0.099; 95%CI, 0.016–0.605; $P = 0.012$), AFP (OR, 2.419; 95% CI, 1.426–4.103; $P = 0.001$), vitamin D (OR, 0.873; 95% CI, 0.801–0.951; $P = 0.002$).

Our findings were confirmed by Hosogaya et al. (23), who reported that bilirubin level is negatively correlated with the IFN therapeutic efficacy as it represents the level of hepatic damage or impairment of its function, however as ALB represents the protein-producing activity of the liver so it is expected to positively correlate with the efficacy of IFN therapy.

Our results are consistent with Di Bisceglie et al. (24), who showed that serum AFP levels decreased significantly during therapy with pegylated interferon α-2a and ribavirin. The decline in AFP with SVR than with relapse further indicates that elevated AFP is more likely to result from inflammation; necrosis and hepatocellular injury. Previous reports have documented a similar pattern of AFP improvement during antiviral therapy of hepatitis C (25). A study done by Wang et al. (26) showed that AST ($P = 0.017$) was found to be significant prognostic factors of interferon response in HCV.

The present work also revealed a significant difference when compared to different stages of fibrosis to different genotypes (Fok1 and Bsm1). These results coincided with that of Baur et al. (27) who stated that VDR gene polymorphism is significantly linked with fibrosis and cirrhosis progression, including the presence of cirrhosis in CHC patients [46].

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