ORIGINAL ARTICLE

Hepatology

Vitamin D Receptor *FokI* Gene Polymorphism Predicted Poor Response to Treatment in Chronic HCV Genotype 4

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Abstract

The aim of this study was to investigate the association between a genetic polymorphism of the vitamin D receptor (VDR) and antiviral responses in Egyptian patients with chronic hepatitis C virus genotype 4 (HCV-4).

Methods: Our study enrolled 100 HCV-4 patients who received pegylated interferon alpha-2a (pegIFNα-*2a) and* ribavirin for 48 weeks. Patients were divided into 2 groups according to their response to therapy: 50 were responders, and 50 were non-responders. All HCV-4 patients were further subjected to the following laboratory tests: HCV-RNA using quantitative PCR, vitamin D level using ELISA and VDR genotype using PCR-RFLP assays, and abdominal ultrasonography.

Results: There was a statistically significant difference in the frequency of the VDR polymorphism (*FokI* rs10735810) between responders (FF:60%, Ff:16%, ff:24%) and non-responders (FF:10%, Ff:26%, ff:64%) (*P<0.001*). There was a statistically significant association between VDR polymorphism with higher ALT levels (ff: 63.2±30.8 U/L, Ff: 48.5±19.5 U/L, FF: 54.4±10.8 U/L, *P=0.04*) and higher alkaline phosphatase levels (ff: 102.6±53.2 U/L, Ff: 100.3±66.4 U/L, FF:68.3±29.4 U/L, *P=0.007*). VDR polymorphism showed no association with baseline vitamin D levels (*P=0.21*).

Conclusion: VDR polymorphism plays a role in the treatment response of HCV and the modification of disease progression in Egyptians infected with chronic HCV-4. **(Int J Biomed. 2016; 6(4):265-270.)**

Key Words: HCV genotype 4 ● response to treatment ● vitamin D ● FokI polymorphism (rs10735810)

Introduction

Chronic HCV remains a worldwide health problem with prevalence rates reaching alarming levels in some areas such as Egypt, where the prevalence is 15%-22%.[1,2] For genotype 4, which is the most prevalent form in Egypt, pegIFNα-*2a* and ribavirin remain the suboptimal "gold standard" for treatment with sustained virologic response rates (SVR) of about 40%-60%.[2] Despite the emergence of novel direct-acting antivirals, to date no interferon-free regimen has proved as effective as interferon-incorporating regimens and none has yet been approved for the treatment of HCV.[3,5]

In the quest to enhance treatment response there has been a relentless search for predictors of response, especially modifiable factors. Recently, the spotlight has been focussed on the role of vitamin D and its relation to progression and response to therapy of HCV.[6] A few studies have recently reported the association of serum vitamin D levels with fibrosis levels and response to treatment in genotypes 1,2,3 HCV.[7-9] Some other studies, however, have negated such evidence.[10] Two small trials have reported an improved SVR with vitamin D supplementation.[11,12] Vitamin D is known to have many immunemodulatory roles and possibly roles in modulating the process of fibrogenesis.[13,14] Immune-regulatory actions of vitamin D are thought to be exerted through the nuclear VDR, expressed in antigen-presenting cells and activated T cells.[13-15] VDR determines interference and/or direct interaction with vitamin D responsive elements in the promoter regions of cytokine genes. Analogous to IL28 gene polymorphisms, it was proposed that genetic polymorphisms affecting the vitamin D pathway may significantly affect response to therapy. Only a few studies have addressed a limited number of genetic polymorphisms where *the vitamin D 1α-hydroxylase* (CYP27B1-1260**)** promoter polymorphism and VDR polymorphisms (rs1544410, rs7975232 and rs731236) have shown significant association with response in

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genotypes 1,2 and 3. [8,16,17] The VDR *FokI* gene polymorphism has been implicated in many immunologic processes including breast and prostate cancer, autoimmune hepatitis, primary biliary cirrhosis, and TB;[6,18-20] its role in HCV is yet to be explored.

Our study aimed to assess the relation between VDR genetic polymorphism (*FokI* rs10735810) and response to therapy in HCV-4 patients. In addition, we also assessed the inter-relation between vitamin D levels and treatment response.

Materials and Methods

Patients

This is a retrospective study assessing the stored sera and whole blood samples of HCV-4 patients who received pegIFNα-*2a* and ribavirin. Fifty consecutive responders (RS) and 50 consecutive non-responders (NR) were selected for the study. Inclusion criteria included: treatment-naïve patients who received pegIFNα-*2a* (180 ug weekly) and weight-based ribavirin (1000-1200 mg daily) for 48 weeks according to current guidelines, compliance with treatment as defined by receiving ≥80% of drugs especially during the first 12 weeks of treatment, written consent of genetic testing and the presence of pre-stored serum and whole blood samples collected during the month before therapy and at the end of therapy. Patients without the previous criteria or with any of the following criteria were excluded: vitamin D or calcium supplementation during therapy or during the 6 months previous to therapy, bone and rheumatologic disorders and renal disorders of any severity. All patients had liver biopsies taken prior to initiation of treatment, and fibrosis was graded according to the METAVIR model. Clinical and demographic characteristics including age, sex, liver biopsy data, HCV viral load, hematologic indices and clinical biochemistry data were extracted from clinical databases.

Analysis of blood samples

A 5 ml whole blood sample was divided into 2 parts. The first part was subjected to DNA extraction and followed by assessment of VDR polymorphism rs10735810 (VDRP) by PCR-*RFLP* analysis. The second part was centrifuged at 3000xg to separate plasma for further assessment of vitamin D level (ng/mL) and quantitative HCV-RNA. Other laboratory measurements were assessed: CBC, INR, blood glucose, ALT, AST, albumin, bilirubin, alkaline phosphatase, α feto-protein, and creatinine.

PCR-RFLP analysis

Total DNA was isolated from mononuclear cells (MNC) using the extraction kit (Qiagene, USA) according to instructions of manufacturer. VDRP rs12979860 genotyping was assessed by RFLP-PCR method, EzWayTM Direct Taq PCR Master mix (Koma Biotech Inc., Seoul, Korea) in 25 μL reaction volume. The primers used for PCR-RFLP were Forward 5`-AGCTGGCCCTGGCACTGACTCTGCTCT-3`and Reverse 5`- ATGGAAACACCTTGCTTCTTCTCCCTC-3` (gene bank accession number: NG008731.1). The thermal cycling profile involved denaturation at 94°C for 15 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec for 35 cycles.

Final extension was continued at 72°C for 10 min. The PCR products were separated by 2% agarose gel electrophoresis. 10uL of the PCR products were digested with 1 unit of the *FokI* restriction endonuclease (New England Biolabs, Hitchin, UK) in a total volume of 20uL at 37°C overnight. Both homozygous (FF and ff) and heterozygous genotypes (Ff) were estimated on 4% agarose gel.

Vitamin D serum level assessment

Vitamin D serum level was detected by ELISA 25-OH Vitamin D kit according to instructions of manufactures (DRG, international Inc., USA). The data are expressed as ng/mL. Grading of vitamin D levels was done as follows: normal (≥30 ng/mL), insufficiency (>10 - < 30 ng/mL), and deficiency (≤10 ng/mL).

qRT-PCR of HCV RNA

The AgPath-ID™ One –Step RT-PCR kit was obtained from Applied Biosystems (Foster City, CA, USA). HCV PCR was performed with lower limit of detection 15 IU/mL.

Statistical analysis

Data were coded and entered using the statistical package SPSS version 22. The odds ratio (OR) and 95%CI were calculated to estimate the strength of associations between each genotype and alleles and patients and controls. *P* values were considered significant when *P*< 0.05.

Results

Patient characteristics are shown in Table 1.

Table 1. Baseline characteristics of patients

Variable	NR	RS	P
Sex Females (%)	28 (56)	12 (24)	0.001
Age, y	42.9±9.5	40.1±11.5	0.188
AST, IU/L	57.7±35.9	44.6±13.6	0.018
ALT, IU/L	61.5±31.2	52.6±10.1	0.060
Alk. Phosphatase, IU/L	113.0±60.2	67.2±26.6	<0.01
Bilirubin, g/dL	0.8±0.3	0.7±0.2	0.139
Albumin, g/dL	4.2±0.7	4.1±0.5	0.137
Creatinine, mg/dL	0.9±0.2	0.9±0.3	0.720
INR	1.1±0.1	1.1±0.1	0.702
WBC count/mL	5.9±1.5	6.6±2.0	0.044
Platelets /mL	223.6±67.4	273.0±94.7	0.003
	11.0±23.4	3.0±2.0	0.020
	F1: 14	F1: 14	0.819
	F2: 29	F2: 31	
	F3: 7	F3: 5	
HCV RNA, IU/mL x10³	1288±2288	464±416	0.015
Vitamin D level, ng/mL	7.26±3.93	5.31±2.83	0.005

All HCV-4 patients suffered vitamin D deficiency or insufficiency (<30 ng/mL) prior to treatment. Although all patients were either deficient or insufficient in vitamin D at baseline, responders had a significantly lower baseline vitamin D level than non-responders (5.31±2.83 ng/mL vs. 7.26±3.93 ng/mL, *P=0.005*). There was a trend for higher baseline

vitamin D level in higher fibrosis stages (F1: 5.5±2.6 ng/mL, F2: 6.2±3.7 ng/ml, F3: 8.2±4.2 ng/mL; F1 vs. F3: *P=0.03*, F1 vs. F2: P=0.08, F1 vs. >F1: *P=0.19*).

At the end of treatment, serum vitamin D levels remained unchanged in non-responders (7.2±2.9 ng/mL vs 6.5±4.8 ng/mL at baseline and end of treatment respectively, *P=0.3*), while in responders serum vitamin D levels improved significantly (5.3±2.8 ng/mL vs 65.8±16.2 ng/mL at baseline and end of treatment respectively, *P<0.001*), Figure 1. According to standard cut-off values, all 50 responders reached normal vitamin D levels (> 30 ng/mL) at the end of treatment while among non-responders only 1(2%) patient improved from being deficient to insufficient and none reached normal levels (*P<0.01*, Table 2).
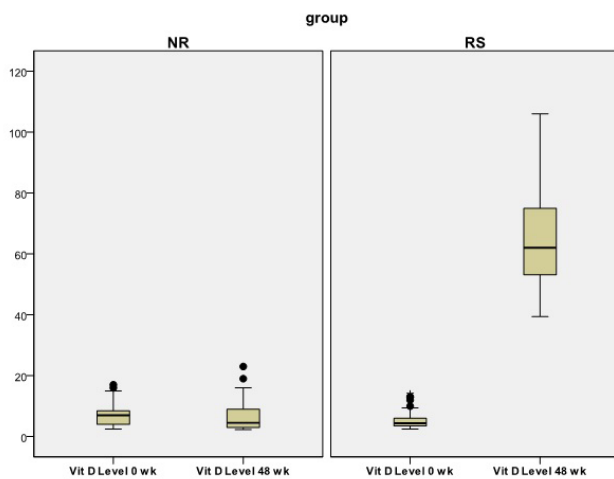

Fig. 1. *Vitamin D level in different studied groups*

Table 2. Vitamin D status before and after treatment

Vitamin D status	NR	RS	P value
Before treatment			0.25
Deficient	41	45	
Insufficient	9	5	
Normal	0	0	
After treatment			<0.001
Deficient	40	0	
Insufficient	10	0	
Normal	0	50	

VDRP

Of the 100 studied patients, 35 had the FF *FokI* variant, 44 had the polymorphic ff variant, and 21 were heterozygous Ff. Of the non-responders, 45(90%) patients had VDR FokI polymorphism, whether homo- or heterozygous (ff or Ff), in comparison to only 20(40%) patients in responders (*P<0.001*, Table 3). When analysing separate allelic combinations, 32(64%) patients with ff genotype were found in non-responders vs. only 12(24%) in responders (*P<0.001*), non-responders included 13(26%) patients with Ff genotype vs 8(16%) in responders (*P=0.22*). The FF variant was much

more prevalent in responders than in non-responders: 30(60%) vs. 5(10%) (*P<0.001*), Figure 2.

Table 3. VDR genotype in responders and non-responders

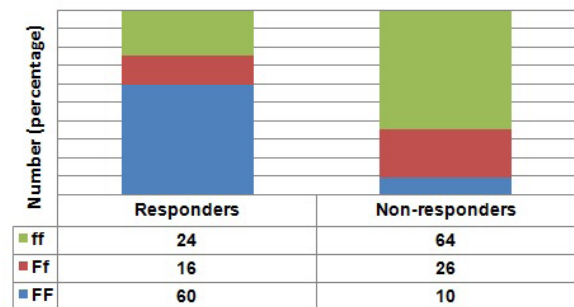| Genotype	NR	RS	P value
ff	32 (64%)	12 (24%)	<0.001
Ff	13 (26%)	8 (16%)	0.22
FF	5 (10%)	30 (60%)	<0.001
(ff + Ff)	45 (90%)	20 (40%)	<0.001

Fig. 2. *Proportion of different VDR FokI genotypes in RS and NR. Numbers indicate % of patients; P<0.001*

Correlation of VDRP with baseline patient characteristics and laboratory data revealed that polymorphism was associated with higher ALT (ff: 63.2±30.8 U/L, Ff: 48.5±19.5 U/L, FF: 54.4±10.8 U/L; *P=0.04*) and alkaline phosphatase (ff: 102.6±53.2 U/L, Ff: 100.3±66.4 U/L, FF: 68.3±29.4 U/L; *P=0.007*) levels. VDRP showed no significant association with fibrosis levels and baseline vitamin D levels; however, there was a trend toward higher vitamin D levels in patients with homozygous polymorphism (ff) versus patients with the wild FF genotype (7.0±3.7 ng/mL vs. 5.6±3.2 ng/ml, *P= 0.08*).

PCR products restricted with *FokI* were shown in Figure 3 and 4. The bands were shown after treatment with *FokI* as FF homozygous (266 bp), Ff heterozygous (266 bp and 193 bp) and ff homozygous (193 bp and 73 bp) according to restriction patterns.

Fig. 3. *Agarose gel electrophoresis showed FokI restriction patterns of the various genotypes of VDR.*

Lane M: DNA ladder (100,200,……bp)
Lane 1-3: 266 bp *PCR* products before treatment of enzyme *(FF genotype)*
Lane 4 and 5: 266 bp *PCR* products after treatment of enzyme *(FF genotype).*
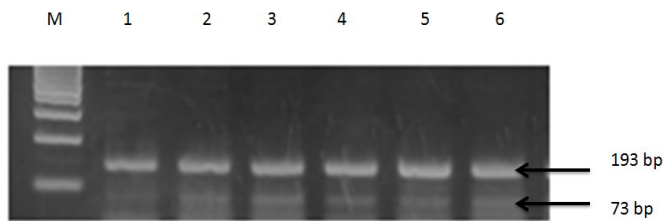Lane 6-9: 266, 193 and 73 bp *PCR* products after treatment of enzyme *(Ff genotype).*

Fig. 4. *Agarose gel electrophoresis showed FokI restriction pattern of ff genotype of VDR.*

Lane M: DNA ladder (100,200,......bp)
Lane 1-6: 193 and 73 bp PCR products after treatment of enzyme *(ff genotype)*.

Predictors of response

Multivariate analysis revealed that the following factors were associated with response (Table 4): lower age, alfa-fetoprotein, baseline vitamin D level, and absence of VDRP.

Table 4.

VDR polymorphism multivariate analysis with other predictors of antiviral response in HCV patients

Variable	OR	95% CI	P value
Age	1.1	1.0-1.3	0.008
Female gender	16.0	2.6-100	0.003
AFP	1.6	1.1-2.4	0.02
Baseline Vitamin D	1.4	1.0-2.0	0.02
VDR polymorphism	14.2	2.0-100	0.008

Discussion

The principle findings of our study are: a) the *FokI* VDRP is independently associated with poor response to therapy in HCV-4; b) chronic HCV-4 patients have a high prevalence of severe vitamin D deficiency; c) responders to treatment of HCV-4 have a remarkable improvement in serum vitamin D levels while non-responders do not; d) baseline vitamin D level does not correlate with a better response to treatment.

Immune-regulatory actions of vitamin D are thought to be exerted through the nuclear VDR, expressed in antigen-presenting cells and activated T cells. [13,14] VDR determines interference and/or direct interaction with vitamin D responsive elements in the promoter regions of cytokine genes. Immune-regulatory effects of vitamin D occur through many mechanisms, including the down-regulated expression of MHC class II, co-stimulatory molecules, and IL-12.[21,22] On the other hand, vitamin D enhances IL-10 production and promotes dendritic cell apoptosis.[21,22] A few studies have recently explored the impact of genetic polymorphisms affecting the vitamin D pathway on the course of chronic HCV and its response to treatment.[8,16,17,23] The CYP27B1-1260 promoter polymorphism (responsible for production of 1,25-dihydroxyvitamin D) has been shown to be a predictor of poor response to treatment in genotypes 1,2 and 3,[8] especially in difficult- to-treat patients.[16,23] These results have been negated, however, by 2 recent large studies that revealed no association between CYP27B1-1260 polymorphism and response to treatment in predominantly genotype 1 cohorts of patients.[17,24] Baur et al. reported that the bAt(CCA) haplotype was associated with impaired response to HCV treatment, a finding that was confirmed by Garcia-Martin et al.[17,24] A single nucleotide polymorphism of the VDR gene (rs2228570 T/C) also had a negative impact on treatment response.[17] The VDR *FokI* polymorphism restriction site is located on exon 2 in the 5′ coding region of the gene.[25] The *FokI* polymorphism has been correlated with many immunologic processes including cancers such as breast, prostate and colorectal cancer, autoimmune hepatitis and TB.[6,23-25] To our knowledge only one study has assessed the role of VDRP in HCV, in a mixed population including genotypes 1,2 and 3, VDRP showed no significant association with treatment response. [8] Our study is the first to assess the effect of VDRP in HCV-4. Our results show a significant and independent negative impact of VDRP on treatment response where 90% of non-responders were carriers of the restriction allele (f) in comparison to only 40% of responders (*P<0.001*). This significance was maintained in multivariate analysis (*P=0.008*). The impact of *FokI* polymorphism on response to treatment is plausible in view of the profound effects it has on the intracellular pathway of vitamin D signalling. VDR whose function is impaired by *FokI* polymorphism determines interference and/ or direct interaction with vitamin D responsive elements in the promoter regions of cytokine genes.[26]

Our results also show an association between VDR *FokI* polymorphism and higher ALT and alkaline phosphatase levels. It might therefore be plausible that vitamin D deficiency or a VDR polymorphism impairing the vitamin D cellular pathway could lead to a proinflammatory milieu and promote hepatic inflammation, which is reflected in elevated ALT levels. This notion is supported by recent studies correlating low serum vitamin D levels with higher inflammatory activity indices in liver biopsies of HCV-infected patients.[7] Even patients with unexplained elevations of ALT have been shown to have lower serum vitamin D levels.[27] The correlation of VDR *FokI* polymorphism with higher alkaline phosphatase levels may also be partly explained by the concept of impairing the anti-inflammatory effects of vitamin D, as over 90% of HCV-infected patients have evidence of bile duct inflammation

Another possible explanation could be the deleterious effect of the VDR polymorphism on bone metabolism, thus increasing bone-specific alkaline phosphatase, although the results of studies testing the association of *FokI* polymorphism with osteoporosis or altered bone turnover have been discordant.[28,29]

All our HCV-4 patients had suboptimal vitamin D levels with the majority being severely deficient (86%). Possible explanations for the association of HCV with vitamin D deficiency have included decreased 25-alpha hydroxylation in the liver, HCV may have the ability to directly suppress 25-alpha hydroxylation through inducing cytokines and

oxidative stresses, and a recent study has shown that HCV alters lipid metabolism directly reducing production of 7-dehydrocholesterol, the precursor of endogenously-produced vitamin D.[30,31] The independent effect of HCV on vitamin D levels is strongly supported by our finding of the remarkable improvement in vitamin D levels after successful eradication of HCV and its persistence in non-responders. Further studies are definitely recommended to depict the exact mechanisms by which HCV alters vitamin D production and metabolism.

Perhaps an unexpected finding in our study was that vitamin D deficiency did not correlate with poor response to treatment. This comes in contrast to many studies that have shown that a lower vitamin D level is associated with poor response to interferon-based treatment in genotypes 1,2 and 3.[7-9] Our study is not, however, the only one to negate such evidence; a recent large study including 274 genotype 1 patients not only demonstrated a lack of association between vitamin D levels and response to treatment, but responders actually had lower baseline vitamin D levels than non-responders in univariate analysis (76.6nmol/L vs. 84.7 nmol/L, respectively; *P=0.03*).[10] Another large study including 310 genotype 1 patients also found no association. [24] In 2 studies that frankly showed no association of response with vitamin D level [our current study and Kitson et al.,2013] vitamin D levels were measured by LC–MS/MS methodology,[10,32] avoiding fallacies that may be induced by other commercially available kits. Another noticeable difference between the studies is the prevalence of advanced fibrosis. In the 3 studies that reported no association of vitamin D with response [our current study, Kitson et al.,2013, and Grammatikos et al.,2011] prevalence of advanced fibrosis was 12%, 14% and 19%, while Bitetto et al.[9] and Petta at al.[7], who both found a relation between vitamin D levels and response, had patients with a high prevalence of advanced fibrosis (29% and 28%).[7,9,10,24] This observation leads us to wonder whether the association of lower vitamin D with a poor response is not strongly confounded by the advanced fibrosis, even though strictly statistically speaking the multivariate analyses in these studies should have excluded this possibility.

Our study has some limitations. First is the retrospective nature of the study. Second, we had no standardization of the time (season) when samples were taken, which could influence vitamin D levels. Third, our cohort consisted of only Egyptian patients and thus results could not be extrapolated to other populations, especially because ethnic differences have been shown in the allelic frequencies in VDR polymorphisms.[33] Fourth, our study design also put some limitation on our interpretation of results; we started by selecting a fixed number of responders and non-responders (limited by samples availability) and compared them, rather than starting with a single cohort of patients and comparing all responders and non-responders. Strictly statistically speaking this approach did not allow us to express the SVR rate in each genetic variant, but rather the prevalence of each genetic variant in both responders and non-responders. Nevertheless, with the obvious high prevalence of the f allele in non-responders, it could be reasonably and confidently deduced

that the f allele is strongly associated with a lower SVR. Fifth, our study did not assess IL28 gene polymorphisms, which have a strong impact on response.

In conclusion, the VDR *Fok*I gene polymorphism is associated with poor response to treatment in HCV-4 patients. Our study reveals significant vitamin D deficiency in HCV-4 patients that reverts with eradication of HCV, yet there is no association of vitamin D levels with response. A larger study assessing the FokI polymorphism, IL28 polymorphism, and vitamin D levels and possibly the effect of vitamin D supplementation is warranted.

Competing interests

The authors declare that they have no conflict of interest.

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