The role of vitamin D receptor polymorphisms in the course of chronic hepatitis C infection

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Abstract

Background Vitamin D and its receptor (VDR) exert important immunoregulatory functions that contribute to liver homeostasis. The aim of this study was to investigate the influence of FokI, ApaI, BsmI and TaqI VDR polymorphisms on cirrhosis development and laboratory variables in patients with chronic hepatitis C (CHC).

Methods A total of 48 patients were enrolled in this retrospective, observational study and underwent genotype analysis; their medical records were examined to obtain relevant data.

Results The cumulative rate of progression to cirrhosis during the course of CHC was 31.3% after a median period of 11 years from diagnosis. Importantly, in multivariate analysis, FokI ff (adjusted hazard ratio [aHR] 13.6, 95% confidence interval [CI] 2.51-73.73; P=0.002) and ApaI aa (aHR 4.69, 95%CI 1.13-19.43; P=0.033) genotypes were independently associated with progression to cirrhosis. The presence of the aa genotype was also associated with higher liver stiffness measurements measured by transient elastography compared to the AA/Aa genotype (12.3kPa interquartile range [IQR] 9.6-17.3 vs. 7.1kPa IQR 5.6-11.1; P=0.012). In addition, higher HCV RNA and lower serum albumin levels were observed in patients with the tt genotype of the TaqI polymorphism compared to TT/Tt carriers, and in patients with the aa genotype compared to AA/Aa carriers. In haplotype analysis, no association was found between any haplotype and disease progression.

Conclusions In patients with CHC, laboratory parameters are influenced by VDR polymorphisms and the development of cirrhosis is related to homozygosity for the dominant trait of ApaI and FokI variants.

Keywords Chronic hepatitis C, vitamin D receptor, gene polymorphisms, liver cirrhosis, fibrosis progression

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Introduction

The introduction of direct-acting antivirals (DAAs) has revolutionized the treatment of hepatitis C virus (HCV) infection by achieving unprecedented rates of viral clearance and setting a paradigm for the successful elimination of chronic viral infections [1,2]. However, the global prevalence of HCV is estimated at 1%, which means that approximately 70 million people remain at risk for potential liver-related complications, including cirrhosis and hepatocellular carcinoma (HCC), due to chronic liver inflammation [3,4]. Importantly, the natural history of HCV infection seems to be determined by a sophisticated interplay between the host's genetic background and immunological responses, in addition to the viral factor. Indeed, spontaneous clearance of infection and response to treatment are influenced by a number of genetic polymorphisms [5-7]. The vitamin D receptor (VDR) belongs to the nuclear receptor superfamily and functions as a ligand-dependent transcription factor. In association with hormonally

active vitamin D, the VDR modulates the expression of more than 900 genes [8]. Beyond regulating calcium homeostasis to maintain bone integrity, the pleiotropic effects of the 1,25(OH)2 vitamin D-VDR complex include neuroprotection, immune modulation, reduction of oxidative stress and regulation of cell proliferation and differentiation [9]. The discovery of the VDR as an important orchestrator of innate and adaptive immunity has sparked interest in elucidating its role in infectious and autoimmune diseases [10,11]. Indeed, recent data have shown the genetic association of certain VDR variants with various liver diseases, such as primary biliary cirrhosis (PBC), autoimmune hepatitis (AIH), nonalcoholic fatty liver disease (NAFLD), and hepatitis B virus infection [12-16]. With respect to HCV infection, certain VDR polymorphisms appear to influence the progression of liver fibrosis, response to interferon-based therapies, and development of HCC [17-20].

FokI, BsmI, ApaI, and TaqI represent the most studied naturally occurring single nucleotide polymorphisms (SNPs) of the *VDR* gene, located on chromosome 12q13.11 and comprises 11 exons, 3 of which (1A, 1B and 1C) encode fragments of the 5'-untranslated region (UTR) and are amenable to differential splicing [21]. The FokI polymorphism is located at exon 2 and is characterized by a T-to-C transition that signifies the loss of the start codon and generates a VDR isoform that is 3 amino acids shorter but more active [22]. The BsmI and ApaI polymorphisms are located within intron 8, whereas TaqI is located downstream within exon 9 [21]. These SNPs do not alter the sequence of the encoded VDR protein, but form a cluster of interconnected polymorphisms that may alter gene expression by interference with intronic regulatory elements or the mRNA splicing process. In addition, the proximity of the cluster to the 3'-UTR could result in modulation of VDR expression by altering mRNA stability [23]. In this study, we investigated the impact of the above VDR genetic variants on demographic, clinical, and laboratory findings and retrospectively examined their association with the clinical progression to cirrhosis in patients with chronic hepatitis C (CHC).

Patients and methods

Study design

This was a single-center observational cohort study involving 48 consecutive patients with CHC who visited our outpatient clinic (Hepatology Clinic, Patras University Hospital) intending to be registered for treatment with DAAs according to the guidelines of the Greek National Health System [24]. Data collection was conducted from October 2018 to March 2019 and included viremic patients with established chronic HCV infection, as evidenced by positive anti-HCV antibodies for at least 6 months and detectable serum HCV RNA. The exclusion criteria were: 1) age <18 years; 2) unwillingness to give informed consent; 3) positivity for serum hepatitis B surface antigen; 4) HIV coinfection; 5) autoimmune liver disease; and 6) chronic liver disease of other cause. All study participants gave informed written consent before enrolment in the study. The study protocol conformed to the ethical principles of the Declaration of Helsinki and was approved by Patras University Hospital Scientific Review Board and Ethics Committee.

Clinical and laboratory evaluation

A comprehensive medical history together with epidemiological and demographic characteristics were obtained from all patients at enrollment. In addition, clinical, laboratory and treatment history data were obtained from the patients' medical records. Liver stiffness measurements were recorded using transient elastography (TE) (Fibroscan®; Echosens). Time intervals between HCV diagnosis, progression to cirrhosis and inclusion in the study were calculated. Alcohol abuse was defined as daily consumption of more than 30 g and 20 g for men and women, respectively [25]. The diagnosis of cirrhosis was based on solid clinical, biochemical, radiological or histological findings [26]. A value of 12.5 kPa was used as the cutoff value for the diagnosis of cirrhosis [27].

VDR genotyping

TaqMan SNP Genotyping Assays (Applied Biosystems) was used to perform genotyping analysis. PCR reactions were performed in MicroAmp® Fast Optical 96-well Reaction Plates (Applied Biosystems) on the Step One Plus real-time PCR system (Applied Biosystems). The rs731236 (TaqI), rs1544410 (BsmI), rs7975232 (ApaI), and rs2228570 (FokI) probes were designed using pre-designed TaqMan SNP genotyping assays (Applied Biosystems). Two non-template control wells were included on each plate. DNA amplification was performed as follows: 95°C for 10 min, followed by 40 cycles of 92°C for 15 sec, and 60°C for 1 min [28].

Statistical analysis

Continuous variables were expressed as medians and interquartile ranges (IQRs), while categorical data were summarized as counts and corresponding percentages. All comparisons were made using non-parametric tests: Pearson's chi-square test or Fisher's exact test were used to assess differences between categorical variables; the Mann-Whitney *U* test and Kruskal-Wallis test were applied when median scores were compared between 2 and 3 groups, respectively. Pearson's chi-square test was used to evaluate the deviation from Hardy-Weinberg equilibrium for each SNP, i.e., by comparing the observed and expected genotype frequencies. The SHEsis software platform was used to perform pairwise linkage disequilibrium analysis (LD) between VDR variants and haplotype analysis based on the expectation-maximization algorithm [29,30]. Time to progression to cirrhosis from diagnosis was analyzed using the Kaplan-Meier method, the log-rank test, and the Cox proportional hazards model. For non-cirrhotic participants, data were censored at inclusion. Partial residual plots (Schoenfeld residual proportional hazards test) were created and analyzed for each variable to test for non-proportionality. All variables with a

P-value of <0.2 in the univariate analysis were first included in the Cox model and then eliminated using backward selection [31]. Statistical analysis was performed using the statistical software package IBM SPSS version 26.0. The threshold of statistical significance was set at 5% (P≤0.05).

Results

Patient characteristics

The main demographic and clinical characteristics of the studied patients are shown in Table 1. The characteristics of the VDR polymorphisms studied are shown in Table 2. The observed genotype proportions for each SNP in our sample are as expected from Hardy-Weinberg. In one participant, the ApaI polymorphism was not identified, while in another, the FokI polymorphism was not identified.

Distribution of clinical and laboratory variables according to VDR polymorphisms

As shown in Table 3, the presence of cirrhosis at the end of follow up differed significantly among CHC patients carrying different FokI alleles (P=0.038). In this regard, the dominant and co-dominant (heterozygotes vs. all homozygotes) genetic models were found to be statistically significant (P=0.011 and P=0.015, respectively), as shown in Table 4. Liver enzymes, laboratory values and liver stiffness were not affected by FokI polymorphisms. ApaI variants had a significant effect on TE measurements (P=0.036), as aa genotype carriers had greater liver stiffness compared to AA/Aa carriers [12.3 kPa (9.6-17.3) vs. 7.1 kPa (5.6-11.1); P=0.012]. In addition, aa genotype was associated with higher HCV RNA (P=0.033) and lower albumin (P=0.034) levels. The variables studied were not affected by BsmI polymorphisms. Regarding TaqI variants, tt genotype carriers had lower albumin (P=0.026) and higher HCV RNA (P=0.021) levels compared to TT/Tt carriers. None of the VDR polymorphisms had an effect on liver function tests, lipid profile and platelet count.

Event analysis

At the time of diagnosis, none of the participants was cirrhotic. The cumulative rate of cirrhosis development during the course of CHC was 31.3% after a median period of 11 years (3-16) from diagnosis. Fig. 1 shows the Kaplan-Meier comparative plot of the cumulative incidence of cirrhosis between the homozygous participants for the dominant trait and the recessive allele carriers for the SNPs FokI and ApaI, respectively. The ff genotype carriers developed cirrhosis over a median period of 12 years (9.8-14.2) from diagnosis, whereas the corresponding period for FF/Ff carriers was 17 years (15.5-18.5) (P=0.01). Similarly, aa genotype carriers developed cirrhosis in a significantly shorter period compared to AA/Aa carriers: 14 years (9.7-18.3) vs. 17 years (12.9-21.1); P=0.021. Age, sex, body mass index, alcohol abuse, and VDR polymorphisms were included in Cox proportional

Table 1 Patient characteristics

Characteristic	Value
Age, years	50 (38-60)
Sex, male, n (%)	30 (62.5)
Race	White
IFN-experienced, n (%)	7 (14.6)
Smoker, n (%)	35 (72.9)
Mode of transmission, n (%)	
IVDU	27 (56.2)
Sexually	8 (16.7)
Parenteral	6 (12.5)
Unknown	7 (14.6)
Genotype, n (%)	
1	14 (29.2)
2	2 (4.2)
3	23 (47.9)
4	9 (18.7)
BMI, kg/m²	25.8 (21.4-28)
ALT, IU/mL	80 (43-197)
AST, IU/mL	50 (33-118)
GGT, U/L	40 (27-127)
ALP, U/L	76 (62-94)
Platelet count, ×10⁹/L	202 (157-272)
Total bilirubin, mg/dL	0.7 (0.5-1)
Glucose, mg/dL	100 (81-115)
Albumin, g/dL	4.5 (4.1-4.6)
Total cholesterol, mg/dL	169 (143-192)
Triglycerides, mg/dL	84 (59-115)
HCV RNA, IU/mL ×10³	259 (66-753)
Liver stiffness, kPa	7.7 (6.2-12.4)

Quantitative values are presented as median (interquartile range)

IFN, interferon; IVDU, intravenous drug user; BMI, body mass index; AST aspartate aminotransferase; ALT, alanine aminotransferase; GGT, γ-glutamyltransferase; ALP, alkaline phosphatase; HCV, hepatitis C virus

hazards regression analysis to estimate the risk of developing cirrhosis for each factor. For non-cirrhotic patients, data were censored at the time of inclusion in the study. In the univariate model, advanced age (P=0.042), homozygosity for the dominant trait FokI (P=0.023), and homozygosity for the dominant trait ApaI (P=0.036) were significantly correlated with progression to cirrhosis (Table 5). In multivariate analysis, after adjusting for confounders, the only independent factors contributing to cirrhosis progression were FokI ff genotype (adjusted hazard ratio [aHR] 13.6, 95% confidence interval [CI] 2.51-73.73; P=0.002) and ApaI aa genotype (aHR 4.69, 95%CI 1.13-19.43; P=0.033).

LD and haplotype analysis

Pairwise LD and VDR haplotype association with progression to cirrhosis in CHC were evaluated using SHEsis software. A very strong LD (D'>0.8) was observed

between the BsmI and ApaI, BsmI and TaqI, and ApaI and TaqI polymorphisms, as shown in the Supplementary

Fig. 1. FokI and ApaI were in strong LD (D'>0.6), while moderate LD was observed between the SNPs FokI

Table 2 Testing genetic markers for compliance with HWE

SNP name	SNP ID	SNP location	Nucleotide change	Codification of SNP alleles	χ2 (sig)*	Compliance with HWE
Fokl	rs2228570	Exon 2	C > T	F > f	0.009 (P>0.05)	Yes
Apal	rs7975232	Intron 8	A > C	A > a	1.945 (P>0.05)	Yes
Bsml	rs1544410	Intron 8	A > G	B > b	0.416 (P>0.05)	Yes
Taql	rs731236	Exon 9	C > T	T > t	0.281 (P>0.05)	Yes

*Deviation from HWE was estimated using the chi square test with one degree of freedom

SNP, single nucleotide polymorphism; HWE, Hardy-Weinberg equilibrium

Table 3 Distribution of clinical and laboratory variables according to VDR polymorphisms

Fokl

	FF	Ff	ff	P-value
Liver cirrhosis, n (%)				
Yes	3 (6.4)	3 (6.4)	9 (19.1)	0.038
No	3 (6.4)	19 (40.4)	10 (21.3)	
Median (IQR)				
ALT, IU/mL	123 (62-156)	70 (49-95)	86 (31-189)	0.691
AST, IU/mL	90 (39-166)	50 (34-77)	54 (28-115)	0.474
GGT, U/L	112 (20-206)	36 (27-54)	46 (28-122)	0.404
ALP, U/L	75 (59-97)	68 (66-80)	87 (61-99)	0.301
Platelet count, ×10⁹/L	179 (147-259)	209 (156-247)	200 (165-276)	0.746
Albumin, g/dL	4 (3.5-4.2)	4.5 (4.3-4.6)	4.4 (4.1- 4.7)	0.13
Total cholesterol, mg/dL	174 (161-185)	169 (140-209)	167 (146-183)	0.935
Triglycerides, mg/dL	129 (75-181)	68 (59-85)	86 (78-116)	0.16
HCV RNA, IU/mL ×10³	3486 (70-13000)	259 (67-697)	201 (122-510)	0.33
Liver stiffness, kPa	7.8 (6.9-17.3)	7.6 (5.6-11.9)	10.2 (6.4-15.2)	0.654

Apal

	AA	Aa	aa	P-value
Liver cirrhosis, n (%)				
Yes	1 (2.1)	6 (12.8)	7 (14.9)	0.243
No	4 (8.5)	21 (44.7)	8 (17)	
Median (IQR)				
ALT, IU/mL	59 (52-104)	74 (44-160)	115 (54-230)	0.48
AST, IU/mL	33 (27-85)	49 (32-105)	81 (52-166)	0.191
GGT, U/L	40 (33-71)	43 (27-141)	60 (26-133)	0.981
ALP, U/L	70 (63-88)	74 (60-90)	81 (68-97)	0.661
Platelet count, ×10⁹/L	206 (187-230)	202 (167-272)	190 (150-280)	0.997
Albumin, g/dL	4.3 (2.6-5)	4.5 (4.3-4.6)	4.2 (3.5-4.3)	0.096
Total cholesterol, mg/dL	164 (145-170)	169 (140-181)	190 (167-212)	0.323
Triglycerides, mg/dL	72 (70-93)	80 (59-101)	162 (93-179)	0.172
HCV RNA, IU/mL ×10³	247 (200-261)	174 (66-480)	751 (462-4051)	0.104
Liver stiffness, kPa	7.2 (7.1-7.9)	6.7 (5.1-12)	12.3 (9.6-17.3)	0.036

BsmI

	BB	Bb	bb	P-value
Liver cirrhosis, n (%)				
Yes	4 (8.3)	8 (16.7)	3 (6.3)	0.557
No	14 (29.2)	13 (27.1)	6 (12.5)	

(Contd...)

Table 3 *(Continued)*

BsmI

	BB	Bb	bb	P-value
Median (IQR)				
ALT, IU/mL	62 (42-92)	93 (39-202)	115 (54-240)	0.353
AST, IU/mL	45 (27-62)	75 (39-116)	83 (48-185)	0.231
GGT, U/L	31 (27-82)	46 (34-142)	70 (28-136)	0.537
ALP, U/L	69 (62-86)	76 (60-94)	87 (72-99)	0.532
Platelet count, ×10⁹/L	239 (195-275)	186 (156-231)	232 (161-312)	0.275
Albumin, g/dL	4.5 (4.3-4.7)	4.5 (4.2-4.6)	4 (3.5-4.2)	0.16
Total cholesterol, mg/dL	164 (140-195)	170 (147-180)	190 (172-201)	0.61
Triglycerides, mg/dL	79 (68-93)	83 (59-114)	93 (74-146)	0.883
HCV RNA, IU/mL ×10³	259 (58-622)	171 (64-676)	944 (239-9610)	0.264
Liver stiffness, kPa	7.6 (6.6-10.2)	7.8 (5.5-12.3)	12.2 (7-19.4)	0.325

TaqI

	TT	Tt	tt	P-value
Liver cirrhosis, n (%)				
Yes	4 (8.3)	8 (16.7)	3 (6.3)	0.468
No	15 (31.3)	13 (27.1)	5 (10.4)	
Median (IQR)				
ALT, IU/mL	61 (41-92)	93 (52-202)	177 (46-242)	0.208
AST, IU/mL	45 (27-62)	75 (36-116)	112 (56-228)	0.14
GGT, U/L	33 (27-43)	40 (26-142)	70 (63-87)	0.521
ALP, U/L	70 (63-87)	75 (59-94)	92 (85-100)	0.271
Platelet count, ×10⁹/L	235 (193-275)	181 (156-231)	259 (176-312)	0.196
Albumin, g/dL	4.5 (4-4.7)	4.5 (4.2-4.6)	3.8 (3.4-4.1)	0.079
Total cholesterol, mg/dL	164 (140-193)	169 (147-180)	190 (172-201)	0.637
Triglycerides, mg/dL	83 (69-90)	86 (56-122)	93 (74-146)	0.881
HCV RNA, IU/mL ×10³	252 (22-618)	171 (64-676)	1190 (462-13000)	0.067
Liver stiffness, kPa	7.6 (6.3-10)	7.8 (5.5-12.3)	12.5 (9.5-19.4)	0.215

Freeman-Halton extension of Fisher's exact probability test for a 2-rows by 3-columns contingency table analysis and Kruskal-Wallis test were used

ALT, alanine aminotransferase; AST aspartate aminotransferase; GGT, γ-glutamyltransferase; ALP, alkaline phosphatase; HCV, hepatitis C virus
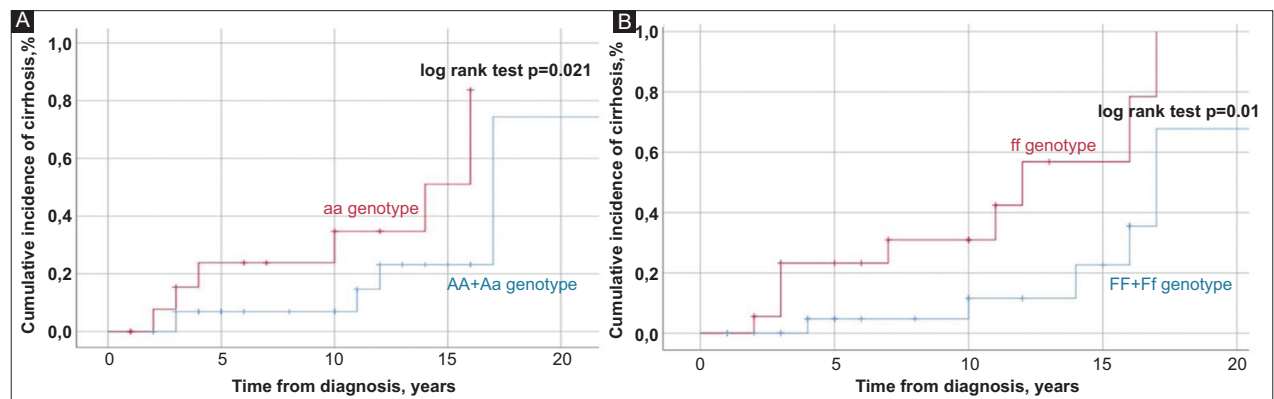

Figure 1 The cumulative incidence of cirrhosis in patients carrying the ApaI aa genotype compared to AA/Aa carriers (A), and in patients with the FokI ff genotype compared to FF/Ff carriers (B)

and BsmI (D'=0.44) and FokI and TaqI (D'=0.41). The estimated distribution of VDR haplotypes in relation to liver status is shown in the Supplementary Table 1. It appears that participants with the BAT haplotype make up the majority of patients (42.4%) who did not develop cirrhosis, suggesting that BAT mediates a possible protective role against disease progression; however, the result was not significant (P=0.246).

Table 4 Comparisons of clinical and laboratory variables between VDR polymorphism according to recessive, dominant, co-dominant and additive genetic models

FokI

	FF vs. Ff+ff	ff vs. FF+Ff	Ff vs. FF+ff	FF vs. ff
Liver cirrhosis*, n	NS	9/19 vs. 6/28; 0.011	3/22 vs. 12/25; 0.015	NS
Albumin, g/dL	NS	NS	NS	NS
HCV RNA, IU/mL ×10³	NS	NS	NS	NS
Liver stiffness, kPa	NS	NS	NS	NS

ApaI

	AA vs. Aa+aa	aa vs. AA+Aa	Aa vs. AA+aa	AA vs. aa
Liver cirrhosis, n	NS	NS	NS	NS
Albumin, g/dL	NS	4.2 (3.5-4.3) vs. 4.5 (4.3-4.7); 0.034	NS	NS
HCV RNA, IU/mL ×10³	NS	751 (462-4051) vs. 201 (63-540); 0.033	NS	NS
Liver stiffness, kPa	NS	12.3 (9.6-17.3) vs. 7.1 (5.6-11.1); 0.012	6.7 (5.1-12) vs. 10.8 (7.1-17); 0.021	NS

BsmI

	BB vs. Bb+bb	bb vs. BB+Bb	Bb vs. BB+bb	BB vs. bb
Liver cirrhosis, n	NS	NS	NS	NS
Albumin, g/dL	NS	NS	NS	NS
HCV RNA, IU/mL ×10³	NS	NS	NS	NS
Liver stiffness, kPa	NS	NS	NS	NS

TaqI

	TT vs. Tt+tt	tt vs. TT+Tt	Tt vs. TT+tt	TT vs. tt
Liver cirrhosis, n	NS	NS	NS	NS
Albumin, g/dL	NS	3.8 (3.4-4.1) vs. 4.5 (4.3-4.6); 0.026	NS	NS
HCV RNA, IU/mL ×10³	NS	1190 (462-13000) vs. 202 (61-614); 0.021	NS	252 (22-618) vs. 1190 (462-13000); 0.046
Liver stiffness, kPa	NS	NS	NS	NS

*Fisher's exact test was used to analyze 2×2 contingency tables and test the association between genotype groups and outcomes (development of cirrhosis or not). The Mann-Whitney U test was used for continuous variables. Quantitative values are presented as median (interquartile range) when the corresponding P-value was ≤0.05

NS, not significant. Aminotransferase, γ-glutamyltransferase, alkaline phosphatase, platelet count, total cholesterol and triglycerides were not significantly different between patients carrying different alleles

Discussion

To the best of our knowledge, this is the first study to suggest that homozygosity for the dominant trait of FokI and ApaI polymorphisms is an independent predictive factor for progression to cirrhosis during the course of CHC. The presence of the aa genotype of the ApaI SNP was associated with greater liver stiffness measurements by TE. In addition,

higher HCV RNA levels and lower serum albumin were found in the patients with the tt genotype of TaqI polymorphism, as well as in those with the aa genotype of ApaI polymorphism. This study supports the view that genetic variants of VDR contribute to the natural history of CHC.

Genetic variants of VDR possibly interfere with vitamin D/VDR-mediated effects and appear to predispose to autoimmune diseases or mediate susceptibility to certain infectious diseases [32,33]. We have previously described

Table 5 Univariate and multivariate analysis of factors associated with risk for cirrhosis development in the course of chronic hepatitis C using Cox regression

Variable	Univariate analysis	HR (95%CI)	Multivariate analysis	aHR (95%CI)
Age, years	0.042	1.06 (1.002-1.1)		
Sex, male	0.486	1.56 (0.45-5.43)		
BMI, kg/m^2	0.603	0.97 (0.85-1.1)		
Alcohol abuse	0.973	1.02 (0.3-3.47)		
FokI, ff[†]	0.023	3.62 (1.2-10.94)	0.002	13.6 (2.51-73.73)
BsmI, BB[‡]	0.119	0.35 (0.1-1.31)		
ApaI, aa[§]	0.036	3.74 (1.09-12.82)	0.033	4.69 (1.13-19.43)
TaqI, TT[¶]	0.109	0.34 (0.09-1.27)		

[†]Reference category: FF+Ff genotype carriers; [‡]Reference category: Bb+bb genotype carriers; [§]Reference category: AA+Aa genotype carriers; [¶]Reference category: Tt+tt genotype carriers

BMI, body mass index; HR, hazard ratio; aHR, adjusted hazard ratio; CI, confidence interval

the contribution of the complex to liver homeostasis and the effects of VDR polymorphisms on inflammatory responses and fibrogenesis in chronic liver disease [28,34,35]. In particular, allelic variations of BsmI and FokI have been associated with the occurrence of PBC and AIH, whereas the BsmI polymorphism has been associated with advanced liver fibrosis in patients with NAFLD [13,36]. In chronic hepatitis B, the FokI ff genotype has been associated with the development of cirrhosis, progression to HCC, and in this case, tumor size and lymph node metastasis [37,38]. With respect to HCV infection, there is evidence that VDR expression is abundant in both hepatic cells and infiltrating inflammatory cells and that VDR reactivity is inversely correlated with the histologic severity of liver disease, underscoring the value of the vitamin D/VDR pathway in this setting [39].

The suggestion that the FokI ff genotype has adverse effects on liver disease progression in CHC is consistent with a previously published prospective study from our center that included patients with various etiologies of cirrhosis and showed that homozygosity for the recessive FokI allele (FF) was an independent prognostic factor for survival in this population [28]. In the same cohort, patients with the FF genotype had reduced interleukin-1β levels, suggesting that the associated short-length VDR leads to a downregulated inflammatory response to chronic injury that is favorable for the liver parenchyma [28]. Homozygosity for the recessive FokI allele (ff) was associated with increased tumor size and higher α-fetoprotein levels in Egyptian patients with HCC (primarily HCV-related), although no association was found between FokI SNP and risk of decompensation [40]. In addition, our results are consistent with a recent study of 554 Thai patients with CHC, in which FokI dominant allele transmission (Ff/ff) was independently associated with advanced liver disease, defined by a fibrosis-4 (FIB-4) score >3.25 [19]. In contrast to our patients, no correlation was found between HCV RNA levels and VDR polymorphisms in the Thai cohort. On the other hand, Scalioni *et al* showed that hepatic fibrosis in a Brazilian HCV-infected population was influenced by BsmI and TaqI SNPs, but not by FokI, while the presence of the ff genotype was associated with normal vitamin D levels, possibly

indicating a smaller risk of severe liver injury [20]. In addition, BsmI and FokI SNPs showed an association with changes in lipid profile, although such a hypothesis is not supported by our results [20]. It should be noted that the above studies used serum biomarkers (FIB-4 or Forns) [19,20], which provide suboptimal diagnostic accuracy for the detection of cirrhosis compared with TE [41], and that they studied patients of different races, facts that may explain to some extent the discrepancies between the results.

Our results are consistent with a number of studies demonstrating that the ApaI aa genotype is associated with progressive liver disease or the development of HCC in CHC [18,42-45], and even claiming that it could serve as a genetic marker to predict the risk of HCC in this setting [43]. Mohammed *et al* showed that an advanced stage of liver cirrhosis, lower serum concentrations of cholecalciferol and the occurrence of HCC were more frequent in patients carrying the dominant (Aa/aa) allele of the ApaI SNP [44]. Consistent with this, Baur *et al* showed that fibrosis progression and cirrhosis, as estimated by histologic scoring methods, were correlated with the presence of the aa genotype, and also demonstrated an additive negative effect of low vitamin D and [CCA] haplotype with respect to liver disease worsening [17]. At the same time, carriers of the aa genotype had higher alanine aminotransferase (ALT) levels and increased matrix metalloproteinase-9 mRNA production, suggesting increased fibrogenesis [17,46]. However, in our analysis, no haplotype was found to be protective or detrimental in relation to cirrhosis development and ALT levels were not associated with VDR polymorphisms. Moreover, a very strong LD between BsmI, ApaI, and TaqI variants was detected in our sample, in agreement with previous data [28,47]. These SNPs might contribute to disease progression in a dependent manner through posttranscriptional regulation of VDR expression. Nevertheless, BsmI, ApaI and TaqI do not cause structural changes in the receptor, so the observed associations may be explained by their linkage to another functional polymorphism elsewhere in the gene.

The propensity of CHC patients with certain VDR polymorphisms to develop cirrhosis at an increased rate suggests a role for the receptor in the pathophysiology of the underlying

fibrosis. Chronic liver injury triggers a relentless wound healing process that leads to hepatic fibrosis characterized by excessive extracellular matrix (ECM) accumulation, eventually leading to cirrhosis. Activation of hepatic stellate cells (HSCs) plays a detrimental role in this scarring process by promoting the deposition of ECM through the production of fibrillar collagens and inhibition of metalloproteinases [48]. Importantly, VDR ligands inhibit transforming growth factor (TGF)-β1/SMAD-dependent activation of HSCs by antagonizing SMAD residence on chromatin, thereby suppressing fibrogenic gene expression [49]. Accordingly, *in vivo* and *in vitro* models of VDR gene knockdown lead to downregulation of vitamin D-mediated opposing effects on TGF-β1 downstream signaling and trigger fibrogenesis [49,50]. In this context, Ito *et al* showed that VDR ligands are capable of suppressing renal tissue fibrosis in animal models through direct blockade of TGF-β/SMAD signal transduction and, more particularly, by inhibiting SMAD3 interaction with target DNA sequences [51]. It could be hypothesized that genetic polymorphisms of the receptor disrupt vitamin D-TGF-β1 crosstalk, either as a consequence of VDR functional modification or via alteration of VDR expression levels. Therefore, impaired vitamin D/VDR interaction may attenuate the inhibitory effect on fibrogenic genes modulated by TGF-β1, thereby accelerating ECM accumulation and hepatic parenchymal deformation. Indeed, profibrogenic mRNA production and serum TGF-β1 levels have previously been associated with VDR polymorphisms [50,52].

The final common pathway of chronic liver disease involves an immune-mediated mechanism of hepatic injury [53]. VDR expression is nearly ubiquitous in immune cell populations, such as T lymphocytes, neutrophils, natural killer cells and antigen-presenting cells (APCs) [54]. The 1,25(OH)2 vitamin D-VDR complex enhances innate immune responses and drives APCs to a more tolerogenic phenotype by inhibiting the maturation of dendritic cells [55,56]. In addition, the vitamin D/VDR pathway alters the Th1/Th2 balance in favor of Th2 differentiation, downregulates Th17 cell development, and promotes T-regulatory cell responses [57]. In this way, appropriate activation of VDR is critical for establishing the balance between anti-inflammatory and proinflammatory cytokine production, and thus for well-orchestrated immune function. The influence of the FokI polymorphism on individuals with HCV-related liver disease could be interpreted in such terms. Specifically, the ff genotype gives rise to the long-VDR isomorph, characterized by reduced activity suppressing the downstream vitamin D-mediated effects [28,58]. However, further studies are needed to elucidate to what extent FokI alleles regulate Th1/Th2 polarization or T-regulatory cell differentiation, as well as how this modulation triggers or abates inflammatory signaling pathways in response to chronic HCV infection.

We acknowledge certain limitations of the current study. First, the sample size was relatively small, which reduced the statistical power of the study; nevertheless, our results are consistent with previous data showing an association between VDR polymorphisms and the development of HCV-related liver complications [18,42-44]. Moreover, accurate assessment of fibrosis status in non-cirrhotic patients to further evaluate the role of VDR in intermediate stages of CHC would require

liver biopsies, which were not available in all our patients. Nonetheless, we analyzed the impact of VDR on a well-defined outcome of CHC, cirrhosis, based on solid evidence. Finally, in most patients the time of exposure to HCV could only be presumed, hence we used the time of diagnosis rather than the time of infection as a starting point for event analysis.

In summary, our results show that FokI and ApaI polymorphisms were independently associated with the occurrence of cirrhosis during the course of CHC. Further studies are needed to establish the causal relationship between VDR polymorphisms and fibrosis progression or cirrhosis development in this setting. Therefore, establishing VDR SNPs as reliable genetic markers would allow identification of patients at increased risk for disease progression and prioritize them for timely treatment.

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Summary Box

What is already known:

- Vitamin D and its receptor (VDR) exert important immunoregulatory effects contributing to liver homeostasis
- Multiple genetic variants of the VDR gene or vitamin D-associated genes have been associated with liver fibrosis progression in patients with liver disease
- Certain VDR gene polymorphisms have been associated with the response to treatment or development of hepatocellular carcinoma among patients infected with hepatitis C virus

What the new findings are:

- Genetic variants of the VDR gene influence laboratory parameters and contribute to the natural history of chronic hepatitis C (CHC)
- Homozygosity for the dominant trait of FokI and ApaI polymorphisms is an independent predictive factor for progression to cirrhosis during the course of CHC
- These findings might help prioritize patients for timely treatment

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Supplementary Table 1 Haplotype frequencies and association with cirrhosis development

Haplotype	Liver cirrhosis, n, (%)	Non-cirrhotic liver disease, n (%)	P-value	OR (95%CI)
bat	12 (42.8)	24 (36.3)	0.733	1.166 (0.48-2.83)
BaT	7 (25)	12 (18.1)	0.557	1.369 (0.48-3.92)
BAT	9 (32.1)	28 (42.4)	0.246	0.581 (0.23-1.46)
baT	0 (0)	2 (3)	0.335	N/A

OR, odds ratio; CI, confidence interval; N/A, not applicable

Supplementary Figure 1 Triangular heatmap visualization of the pairwise linkage disequilibrium pattern in the study sample. Each square represents the D' value and R^2 value between pairs of polymorphisms. The intensity of the color of the blocks illustrates the linkage disequilibrium strength between the vitamin D receptor variants