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ORIGINAL ARTICLE

Genetic variants underlying vitamin D metabolism and VDR–TGFβ-1–SMAD3 interaction may impact on HCV progression: a study based on dbGaP data from the HALT-C study

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Vitamin D deficiency is prevalent in liver disease and vitamin D has been shown to decrease hepatic fibrosis through an anti-TGFβ-1/SMAD3 effect mediated by the vitamin D receptor. Thus, we hypothesized that genetic variants involved in vitamin D metabolism and/or VDR/TGFβ-1/SMAD3 interaction could impact on the progression of chronic HCV. We obtained or imputed genotypes for 40 single nucleotide polymorphisms (SNPs) located in genes implicated in vitamin D metabolism from the HALT-C cohort via dbGaP. The HALT-C study followed 692 chronic HCV patients over 4 years, evaluating clinical outcomes including worsening of fibrosis, hepatic decompensation (gastric/esophageal bleeding, CTP > 7, ascites, spontaneous bacterial peritonitis and encephalopathy), development of hepatocellular carcinoma, and liver death. We tested the selected SNPs for association with these outcomes in 681 HALT-C subjects. Eleven SNPs presented tendency towards significance ($P < 0.05$): four SNPs in *DHCR7* related to with hepatic decompensation (rs4944957, rs12800438, rs3829251 and rs4945008); two in *GC* to worsening of fibrosis and liver death (rs7041 and rs222020); two in *CYP2R1* to ascites and hepatocellular carcinoma (rs7116978 and rs1562902); two in *VDR* to gastric/esophageal bleeding and hepatocellular carcinoma (rs4516035 and rs2239186); and one in *SMAD3* to worsening of fibrosis and encephalopathy (rs2118610). Only rs1800469 in *TGFB1* was statistically associated with hepatic decompensation after Bonferroni's correction ($P < 0.00125$). In conclusion, rs1800469 in *TGFB1* was associated to hepatic decompensation in chronic hepatitis C, while the other 11 described polymorphisms must be evaluated in a larger cohort to determine the possible role of vitamin D in hepatitis C.
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INTRODUCTION

Chronic hepatitis C (CHC) leads to hepatic fibrosis, the liver's wound-healing response to injury.[1] Advanced liver fibrosis results in cirrhosis, which is characterized by distortion of the hepatic vasculature. The major consequences of cirrhosis in the natural course of CHC are impaired liver function, portal hypertension and an increased risk of developing hepatocellular carcinoma (HCC).[2,3] Given the high prevalence of vitamin D deficiency in CHC patients, the role of vitamin D in liver disease has received much attention over the past few years. Indeed, low levels of vitamin D have been found to be associated with a higher likelihood of advanced liver disease.[4]

The majority of vitamin D is synthesized in the skin as a result of exposure to UV radiation. In an initial step, 7-dehydrocholesterol is converted into pre-vitamin D by the 7-dehydrocholesterol reductase, followed by an isomerization step which completes the synthesis of vitamin D. A small proportion of vitamin D is obtained from the diet. Vitamin D from both skin conversion and dietary intake are hydroxylated in the liver by cytochrome P450 enzymes (CYP2R1). 25-hydroxyvitamin D (25-OH VD) circulates bound to vitamin D-binding protein (DBP), which is synthesized in the liver. The 1a-hydroxylation of 25-OH VD by CYP27B1 in the kidney produces 1,25-dihydroxyvitamin D (1,25-OH VD), the form that activates the vitamin D receptor (VDR). The enzyme CYP24A1 is responsible for catabolizing the excess of 1,25-OH VD. The VDR acts as a transcription factor that binds to vitamin D responsive elements in the promoter region of target genes,[5] regulating the expression of >200 genes that influence cell proliferation, differentiation, apoptosis, immunomodulation and angiogenesis.[6]

Among its many actions, it has been described that vitamin D decreases hepatic fibrosis by limiting hepatic stellate cell (HSC)

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Table 1 Polymorphisms of vitamin D metabolism related to serum levels of vitamin and/or CHC progression selected from the literature

Chr	*Gene*	*Protein*	*dbSNP*	*Alleles*	*Position (bp)*	*Reported association*	*Citation*
4	*GC*	DBP	rs17467825	A/G	72605517	Vitamin D serum levels	25,46
			rs2282679	T/G	72608383	Vitamin D serum levels, HCC	22–25,46–48
			rs3755967	T/C	72609398	Vitamin D serum levels	25,46
			rs2298850	C/G	72614267	Vitamin D serum levels	25,46
			rs4588	T/G	72618323	Vitamin D serum levels	23,25
			rs7041	C/A	72618334	Vitamin D serum levels	22,23,25,46
			rs222020	C/T	72636272	Vitamin D serum levels	27,28,49
			rs1155563	C/T	72643488	Vitamin D serum levels	22–25,46
			rs2298849	G/A	72648851	Vitamin D serum levels	27,47
			rs115316390	A/G	72651159	Vitamin D serum levels	46
			rs221999	G/A	72649048	Vitamin D serum levels	30
11	*DHCR7*	DHCR7	rs1790349	T/C	71142350	Vitamin D serum levels	22,23
			rs7944926	A/G	71165625	Vitamin D serum levels	25
			rs12785878	G/T	71167449	Vitamin D serum levels, Liver fibrosis	24,25,50,51
			rs4944957	A/G	71168035	Vitamin D serum levels	25
			rs12800438	A/G	71171003	Vitamin D serum levels	25
			rs3794060	C/T	71187679	Vitamin D serum levels	25
			rs3829251	A/G	71194559	Vitamin D serum levels	22–24
			rs4945008	A/G	71221248	Vitamin D serum levels	25
			rs11234027	A/G	71234107	Vitamin D serum levels	22
11	*CYP2R1*	CYP2R1	rs7116978	C/T	14881771	Vitamin D serum levels	25,29
			rs1993116	G/A	14910234	Vitamin D serum levels, HCC	22,25,46,48
			rs10500804	G/T	14910273	Vitamin D serum levels	25
			rs12794714	A/G	14913575	Vitamin D serum levels	25,27,46
			rs10741657	A/G	14914878	Vitamin D serum levels	25,27,46,52
			rs2060793	A/G	14915310	Vitamin D serum levels	22,23,25,46
			rs1562902	C/T	14918216	Vitamin D serum levels	27,30
			rs10766197	A/G	14921880	Vitamin D serum levels	27,30
12	*CYP27B1*	CYP27B1	rs10877012	G/T	58162085	Vitamin D serum levels	47,52
12	*VDR*	VDR	rs731236	G/A	48238757	Liver fibrosis, HCC	8,10,53
			rs7975232	A/C	48238837	Liver fibrosis, HCC	8,10,53,54
			rs757343	T/C	48239675	Liver fibrosis	8,35
			rs1544410	T/C	48239835	Liver fibrosis, HCC	8,10,53
			rs2239186	G/A	48269410	Vitamin D serum levels	30,32
			rs4516035	C/T	48299826	Liver fibrosis	8
15	*SMAD3*	SMAD3	rs9806504	C/T	67392777	Skin fibrosis (keloid)	33
			rs11071932	A/G	67399546	Skin fibrosis (keloid)	33
			rs2118610	T/C	67428334	Skin fibrosis (keloid)	33
19	*TGFB1*	TGFβ-1	rs1800471	C/G	41858876	Liver fibrosis	55,56
			rs1800469	G/A	41860296	Cirrhosis, HCC	36,37
20	*CYP24A1*	CYP24A1	rs2296241	A/G	52786219	Vitamin D serum levels	30
			rs17219315	A/G	52788446	Vitamin D serum levels	30
			rs73913757	C/T	52790518	Vitamin D serum levels	46
			rs2244719	C/T	52782858	Vitamin D serum levels	30

activation through an anti-transforming growth factor beta 1 (TGFβ-1) effect mediated by VDR.[7,8] Ding *et al.*[7] found 10,436 genomic sites to be co-occupied by both VDR and SMAD3, an effector of TGFβ-1 cascade, thus showing an interaction between the two transcription factors in HSCs. A common genetic variant of the *VDR* gene is the bAt haplotype, which is composed of three polymorphic sites (rs1544410 T/C, rs7975232 A/C and rs731236 G/A).[9] The bAt [CCA]-haplotype has been associated with fibrosis progression rates and cirrhosis.[10] We thus hypothesized that genetic variants involved in vitamin D metabolism (*DHCR7, GC, CYP2R1, CYP27B1* and *CYP24A1*) and/or in the VDR-TGFβ-1-SMAD3 interaction could impact on the evolution of chronic hepatitis C.

MATERIALS AND METHODS

Patient data

The present study is based on genotype and clinical phenotype data from the Hepatitis C Antiviral Long-term Treatment against Cirrhosis (HALT-C) Trial,[11] which we obtained from the Database of Genotypes and Phenotypes (db-GaP; http://www.ncbi.nlm.nih.gov/gap; accession number phs000430.v1.p1).[12] Access to the controlled portion of the dbGaP data were granted by the NIH

Data Access Committee. This study has also been approved by the Ethics Committee of Universidade Federal do Rio Grande do Sul (UFRGS).

The HALT-C Trial is a randomized controlled trial designed to evaluate the safety and efficacy of long-term use of pegylated-interferon (PEG-IFN) for the treatment of chronic hepatitis C in patients who failed to respond to previous interferon therapy. HALT-C genotyping was funded through a Bench-to-Bedside Award (NIH) to Gonzalo Laje, MD in the Human Genetics Branch, Intramural Research Program, NIMH, NIH, U.S.DHHS. The original data set, was submitted to dbGap by Dr Laje.

Information regarding inclusion and exclusion criteria and randomization can be found elsewhere.[11] Briefly, participants were adult CHC patients of any genotype who did not respond to previous interferon treatment, who had advanced fibrosis without hepatic decompensation or hepatocellular carcinoma, and who were free of any other liver diseases. A total of 692 subjects were randomized and followed over 4 years. A clinical and serological evaluation was carried out every 3 months; liver biopsies, endoscopic evaluation of esophageal varices and an assessment of portal hypertension were performed at the time of the enrollment and after 2 and 4 years. Information on the subjects' treatment status is not available through dbGaP. In this study, we evaluated the following outcomes:

(A) Worsening of fibrosis, defined by a 2-point increase in Ishak Score for patients with an Ishak <4;
(B) Hepatic decompensation in cirrhotic patients (Ishak 5 or 6) who presented any of the following: bleeding from gastric or esophageal varices, Child-Turcotte-Pugh Score (CTP) >7, ascites, spontaneous bacterial peritonitis, and hepatic encephalopathy;
(C) Development of hepatocellular carcinoma;
(D) Liver death: need for liver transplant and/or liver-related death.

Genetic variant selection

We selected, in the literature, 44 single nucleotide polymorphisms (SNPs) of vitamin D metabolism (genes *DHCR7, GC, CYP2R1, CYP27B1, CYP24A1)* and VDR–TGFβ-1–SMAD3 interaction that have been previously related to serum levels of vitamin D and/or to HCV progression for investigation in this cohort (Table 1).

Genotype analysis and imputation

Although HALT-C data included genotypes at 617 431 polymorphisms from the Illumina Human610_Quadv1_B platform, we were only interested in analyzing the 44 SNPs described in Table 1. Binary pedigree format files were obtained from db-GaP and handled with PLINK v.1.07.[13] All markers were flipped to forward strand orientation, and ambiguous variants (A/T or C/G) were removed. Individuals with sex chromosome abnormalities and sex inconsistencies, as well as individuals with $<90\%$ genotype data were removed from the data set. SNPs with a genotype call rate $<97\%$ or a minor allele frequency (MAF) <0.01, and SNPs deviating from the Hardy-Weinberg equilibrium ($P<10^{-6}$) were also excluded.

Genotypes were available for 21 of the 44 SNPs listed in Table 1. Genotypes for the remaining 23 were imputed using MaCH-Admix v. 2.0.203[14] in the integrated running mode. As a reference panel, we used a 1000 Genomes vcf file (ALL Phase 3 v.5), containing the haplotypes of 2,504 samples for ~81.2 M polymorphic markers.[15] To speed up the computation, genotypes were imputed one chromosome at time only for chromosomes of interest (4, 11, 12, 15, 19, and 20), and the reference panel was split in chunks of 5 Mb centered around the SNP of interest, with flanks of 250 kb on either side. Imputation running settings includ: states 200, imputestates 1000 and rounds 50. At the end of each run, imputed genotypes were extracted with fcGENE 1.0.7[16] in binary pedigree

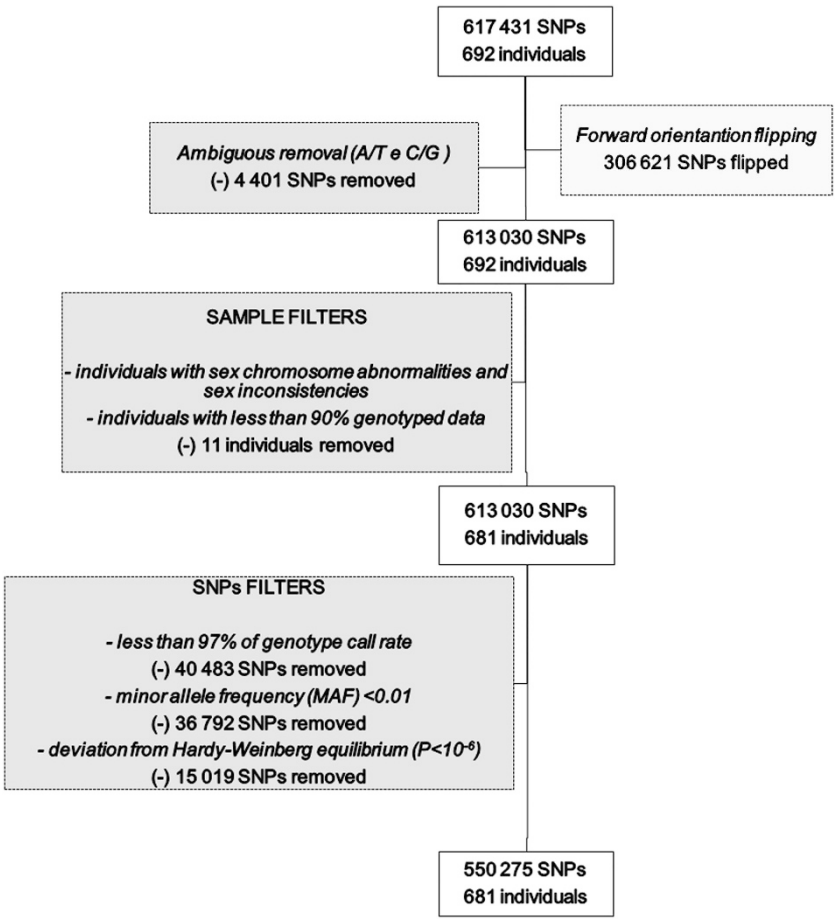

Figure 1 Summary of the quality control procedure, including numbers of individuals and SNPs removed. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

format. The imputation quality was measured by the squared correlation between imputed and true genotypes (Rsq). Variants with an Rsq > 0.5 were considered as successfully imputed.[17] SNPs with a lower Rsq were excluded from the analysis.

Statistical analysis

For each of the four outcomes, a logistic regression analysis was conducted to evaluate the association with the 44 SNPs of interest. The five signs of hepatic decompensation—bleeding of gastric or esophageal varices, CTP score >7, ascites, spontaneous bacterial peritonitis, and hepatic encephalopathy—were analyzed separately by logistic regression, and were considered secondary outcomes. Logistic regression analyses were adjusted for age, sex and race (as a dummy variable—white and non-white) after checking in single-regression analysis if these variables were associated with the studied polymorphisms. Analyses were carried out to test for dominant (minor–minor or minor–major vs major–major), recessive (minor–minor vs minor–major or major–major), and additive models of inheritance. We regard this as an exploratory study; therefore, SNPs reaching the $P<0.05$ threshold were considered as showing 'tendency towards association'. Subsequently, Bonferroni's correction was conducted to adjust for multiple comparisons ($P<0.05$/number of evaluated SNPs in the analyses). Statistical analysis was performed using PLINK. The *VDR* bAt [CCA]-haplotype was evaluated using MaCH-Admix and Haploview v 4.2,[18] and Chi-square tests were performed in SPSS 20.0 (SPSS Inc, Chicago, Il, USA).

RESULTS

After applying quality control filters, 681 individuals were retained. 550,275 SNPs were used for imputation (Figure 1). Four SNPs were removed from the final analyses due to low quality imputation (rsq < 0.5) (Supplementary Table 1), making for a total of 40 SNPs (21 genotyped, 19 imputed). Level of significance after Bonferroni's correction was set at 0.00125 ($P<0.05/40$).

Table 2 Demographic and clinical features of chronic hepatitis C patients

Variable	*Chronic hepatitis C (n = 681)*
Sex (male)[a]	486 (71.4%)
Age (years)[b]	49.9 (±7)
Ethnicity[a]	
White	493 (72.4%)
Black	117 (17.2%)
Hispanic	55 (8.1%)
Other	16 (2.3%)
Baseline Ishak score[a]	
2	47 (6.9%)
3	233 (34.2%)
4	124 (18.2%)
5	150 (22.0%)
6	127 (18.6%)
Cirrhosis decompensation	126/393 (32.1%)
Gastric/esophageal bleeding[c]	10 (2.7%)
CTP > 7	105 (26.7%)
Ascites	59 (15.0%)
Spontaneous bacterial peritonitis	4 (1.0%)
Hepatic encephalopathy	34 (8.7%)
Hepatocellular carcinoma[a]	55 (8.1%)
Mortality[a]	94 (13.8%)
Liver related	52 (55.3%)
Not liver related	36 (38.3%)
Skewed	6 (6.4%)
Transplant[a]	55 (8.1%)

[a]*n* (%).
[b]Mean(±s.d.).
[c]Data available for 373 patients.

Demographic and clinical data of analyzed patients are summarized in Table 2. The majority of patients were male (71.4%) and white (72.4%). Mean age at enrollment was 49.9 years, ranging from 19 to 80 years. Some of the studied SNPs presented association with studied available covariates (sex, age and race) in single regression analysis (Supplementary Table 2).

For the analysis of worsening fibrosis, a total of 366 patients with baseline Ishak ⩽ 4 and at least two liver biopsies to evaluate progression were included (Figure 2). One hundred patients had a minimum 2-point increase in Ishak score during the four-year follow-up. The results of the logistic regression analysis for worsening fibrosis are described in Table 3. Two SNPs in the *GC* gene, rs7041 and rs222020, as well as one SNP in *SMAD3,* rs2118610, presented only tendency towards association under additive and dominant models. As the *VDR* bAt [CCA]-haplotype (rs1544410, rs7975232 and rs731236) has been associated with a worsening of fibrosis, it was analyzed separately. Haplotype frequencies were: 0.468 for CCA; 0.354 for TAG; 0.153 for CAA; and 0.013 for TAA. Haplotype frequencies did not differ between patients who had an increase of at least 2 point in their Ishak score and those who did not. Homozygosity at the bAt [CCA] haplotype versus all other genotypes was not found to be associated to a worsening of fibrosis [23% (23/100) vs 20.7% (55/266); $P=0.363$].

One-hundred and twenty-six of the 393 cirrhotic patients presented at least one of the following: bleeding from gastric or esophageal varices, ascites, spontaneous bacterial peritonitis, hepatic encephalopathy and/or CTP Score > 7. The latter was the most frequent sign, present in 105 patients (Table 2). In the regression analysis for hepatic decompensation, 4 polymorphisms in the *DHCR7* gene (rs4944957, rs12800438, rs3829251 and rs4945008) showed tendency towards significance, and only rs1800469 in *TGFB1* was significant after Bonferroni's correction (Table 4). When each one of the five decompensation variables were analyzed separately, six SNPs demonstrate tendency towards significance, including rs1800469 in *TGFB1* with CTP > 7, but none of these were associated after Bonferroni's correction (Table 4).

After 4 years of follow-up, 55 patients had developed HCC. In the logistic regression analysis of HCC, two polymorphisms showed tendency towards significance under the recessive model, rs1562902 in *CYP2R1* and rs2239186 in *VDR* (Table 5). The overall mortality over the course of the study was of 94 patients (13.8%). Fifty-two of those deaths were liver related. Ninety-nine patients needed a transplant and/or progressed to a liver related death. The polymorphism rs7041 showed tendency towards significance for this outcome under the recessive model of inheritance (Table 5).

A summary of SNPs reaching tendency towards significance or significance after Bonferroni's correction for any given outcome can be found in Table 6. Risk alleles found at rs1800469 for hepatic decompensation, rs4516035 for gastric esophageal bleeding and rs3829251 for hepatic decompensation were in accordance with risk alleles reported in the literature.

Results under the additive model for all 40 SNPs for worsening of fibrosis, hepatic decompensation, HCC and liver death are presented in Supplementary Table 3.

DISCUSSION

The high prevalence of vitamin D deficiency is independent of the etiology of liver disease and may impact upon clinical outcomes.[19]

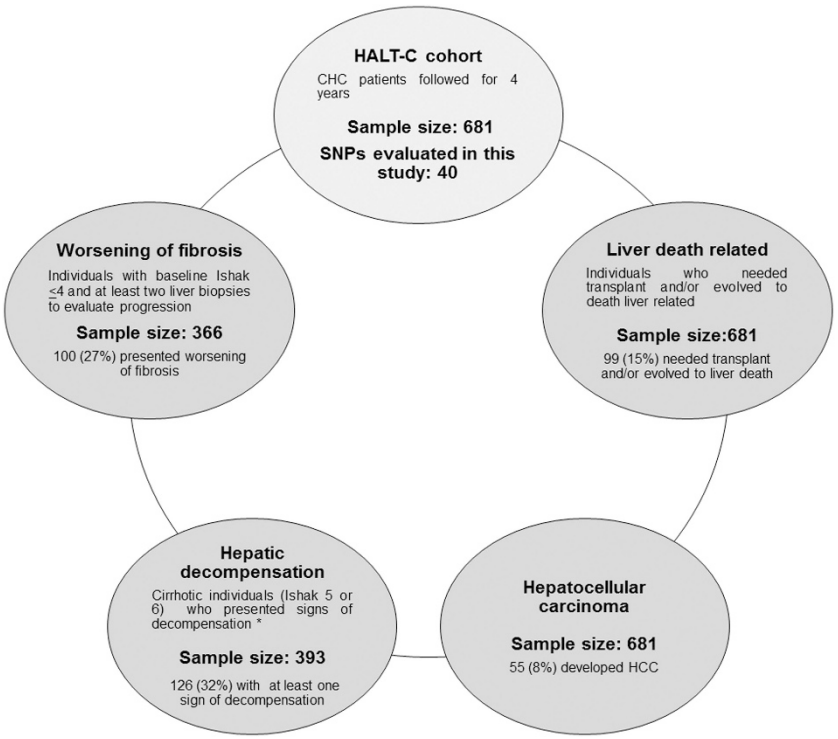

Figure 2 Sample size for each of the analyzed outcomes. *Signs of hepatic decompensation: bleeding of gastric or esophageal varices, Child-Turcotte-Pugh Score >7, ascites, spontaneous bacterial peritonitis, and hepatic encephalopathy. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

Table 3 Polymorphisms with tendency towards association with worsening of fibrosis

Chr	*Gene*	*SNP*	*Minor*	*Model*		*Affecteds* (n)	*Unaffecteds* (n)	*OR*	*95% CI*	P-*value*	*P adjusted*[a]
Worsening of fibrosis (> 2 Ishak points)											
4	*GC*	rs7041	A	Additive	A	0.42 (84)	0.55 (291)	0.59	0.41–0.86	0.004872	0.1949
					C	0.58 (116)	0.45 (241)				
				Dominant	AA+AC	0.64 (64)	0.79 (210)	0.47	0.28–0.80	0.005004	0.2001
					CC	0.36 (36)	0.21 (56)				
4	*GC*	rs222020	C	Additive	C	0.16 (31)	0.27 (142)	0.55	0.35–0.85	0.07682	0.3073
					T	0.84 (169)	0.73 (390)				
				Dominant	CC+CT	0.27 (27)	0.44 (116)	0.50	0.29–0.84	0.009792	0.3917
					TT	0.73 (73)	0.56 (150)				
15	*SMAD3*	rs2118610	T	Additive	T	0.48 (95)	0.38 (202)	1.51	1.07–2.12	0.01806	0.7222
					C	0.52 (105)	0.68 (330)				
				Dominant	TT+TC	0.76 (76)	0.62 (164)	2.06	1.21–3.49	0.007354	0.2942
					CC	0.24 (24)	0.38 (102)				

[a]Bonferroni's correction.

Polymorphisms in *GC*, *CYP2R1* and *CYP27B1* could play a role in treatment response to PEG-IFN and ribavirin,[20] although baseline levels of 25-OH VD do not seem to.[21] With this in mind, we hypothesized that genetic variants involved in vitamin D metabolism could impact on the progression of liver disease in outcomes such as worsening of fibrosis, hepatic, development of HCC and liver death. SNPs in *DHCR7*, *GC* and *CYP* genes are mostly related to vitamin D synthesis and can influence its circulating levels. In addition, variants involved in the VDR/TGFβ-1/SMAD3 signaling cascade could have an effect on fibrosis and other liver-related events.

We tested our hypothesis on data collected by the HALT-C trial, which evaluated the efficacy of long-term use of pegylated-interferon for preventing progression of CHC. We selected 42 SNPs that had previously been reported to be related to progression of HCV-chronic liver disease and two SNPs related to scarring (both in *SMAD3*). After discarding four SNPs because of insufficient imputation quality scores, 12 of these SNPs were found to be associated or with tendency towards association with at least one of the studied outcomes.

Four SNPs in the *DHCR7* gene presented tendency towards significance with hepatic decompensation and/or gastric/esophageal bleeding. Although these findings indicate only a tendency, the A allele of rs3829251, related to the presence of hepatic decompensation, has previously been reported to be associated to lower levels of circulating vitamin D.[22–24] The other three SNPs were linked to serum levels of

Table 4 Polymorphisms with tendency towards association and associated with hepatic decompensation, bleeding from gastric or esophageal varices, Child-Turcotte-Pugh Score > 7, ascites, spontaneous bacterial peritonitis, and hepatic encephalopathy

Chr	*Gene*	*SNP*	*Minor*	*Model*		*Affected* (n)	*Unaffected* (n)	*OR*	*95% CI*	P-*value*	*P adjusted*[a]
Hepatic decompensation											
11	*DHCR7*	rs4944957	A	Dominant	AA+AG	0.54 (68)	0.50 (134)	1.56	1.02–2.38	0.04185	1
					GG	0.46 (58)	0.50 (133)				
11	*DHCR7*	rs12800438	G	Dominant	GG+GA	0.55 (69)	0.51 (136)	1.56	1.02–2.40	0.04115	1
					AA	0.45 (57)	0.49 (131)				
11	*DHCR7*	rs3829251	A	Dominant	AA+ AG	0.34 (43)	0.69 (87)	1.58	1.03–2.44	0.0374	1
					GG	0.66 (83)	0.31 (180)				
11	*DHCR7*	rs4945008	A	Dominant	AA+AG	0.60 (76)	0.55 (146)	1.58	1.01–2.47	0.04309	1
					GG	0.40 (50)	0.45 (121)				
19	*TGFB1*	rs1800469	A	Recessive	AA	0.19 (24)	0.08 (22)	3.22	1.69–6.12	0.000369	0.01475
					AG+GG	0.81 (102)	0.92 (245)				
Bleeding from gastric or esophageal varices											
11	*DHCR7*	rs4944957	A	Recessive	AA	0.30 (3)	0.10 (38)	4.68	1.08–20.18	0.03867	1
					AG+GG	0.70 (7)	0.90 (325)				
11	*DHCR7*	rs12800438	G	Recessive	GG	0.30 (3)	0.12 (43)	4.42	1.01–19.4	0.04926	1
					GA+AA	0.70 (7)	0.88 (320)				
12	*VDR*	rs4516035	C	Additive	C	0.65 (13)	0.38 (273)	2.66	1.08–6.58	0.03394	1
					T	0.35 (7)	0.62 (453)				
Child-Turcotte-Pugh Score > 7											
19	*TGFB1*	rs1800469	A	Recessive	AA	0.18 (19)	0.09 (27)	2.30	1.20–4.41	0.01204	0.4815
					AG+GG	0.82 (86)	0.91 (261)				
Ascites											
11	*CYP2R1*	rs7116978	T	Dominant	TT+TC	0.71 (42)	0.55 (183)	2.08	1.13–3.82	0.01799	0.7196
					CC	0.29 (17)	0.45 (151)				
Hepatic encephalopathy											
15	*SMAD3*	rs2118610	T	Additive	T	0.62 (21)	0.72 (258)	0.57	0.33–0.99	0.04641	1
					C	0.38 (13)	0.28 (101)				

[a]Bonferroni's correction.

Table 5 Polymorphisms with tendency towards association with hepatocellular carcinoma and liver death that showed statistical significance

Chr	*Gene*	*SNP*	*Minor*	*Model*		*Affecteds* (n)	*Unaffecteds* (n)	*OR*	*95% CI*	P-*value*	*P adjusted*[a]
Hepatocellular carcinoma											
11	*CYP2R1*	rs1562902	C	Recessive	CC	0.31 (17)	0.19 (122)	1.86	1.006–3.44	0.04778	1
					CT+TT	0.69 (38)	0.81 (504)				
12	*VDR*	rs2239186	G	Recessive	GG	0.09 (5)	0.03 (18)	2.978	1.036–8.561	0.04278	1
					GA+AA	0.91 (50)	0.97 (608)				
Liver death											
4	*GC*	rs7041	A	Recessive	AA	0.19 (19)	0.28 (165)	0.5589	0.3164–0.987	0.04496	1
					AC+CC	0.81 (80)	0.72 (417)				

[a]Bonferroni's correction.

vitamin D in a single GWAS study, which did, however, not report risk alleles.[25]

In the *GC* gene two polymorphisms showed tendency towards significance with worsening of fibrosis or liver death. SNP rs7041 is one of the two most common polymorphisms of the *GC* gene, leading to an amino acid change[26] and to lower serum levels of vitamin D.[22,25] However, the A allele identified in this study as 'risk', was reported to be responsible for higher levels of vitamin D in the literature. For rs222020, an intron variant of *GC*, that also showed tendency to significance, has been described both as a protective and as a risk factor for vitamin D levels in Caucasians.[27,28]

At the *CYP2R1* locus, two SNPs showed tendency towards significance to ascites and to hepatocellular carcinoma. While we found the T allele at rs7116978 to be weakly related with ascites, it has been reported to be associated to higher vitamin D serum levels.[29] We found that the homozygosity for the C allele of the polymorphism rs1562902, tended towards significance with the development of hepatocellular carcinoma. In previous studies, the C allele has been

Table 6 Comparison of risk alleles identified in the present study to the literature

		Current study		*Literature*		
Gene	*SNP*	*Risk Alelle*	*Effect*	*Risk Alelle*	*Effect*	*Studies*
DHCR7	rs4944957	A	Hepatic decompensation Gastric/esophageal bleeding	–	Vitamin D serum levels	Wang *et al.*,[25]
	rs12800438	G	Hepatic decompensation Gastric/esophageal bleeding	–	Vitamin D serum levels	Wang *et al.*,[25]
	rs3829251	A	Hepatic decompensation	A	Vitamin D serum levels	Ahn *et al.*,[22] Lu *et al.*,[23] Kuhn *et al.*,[24]
	rs4945008	A	Hepatic decompensation Gastric/esophageal bleeding	–	Vitamin D serum levels	Wang *et al.*,[25]
GC	rs7041	C	Worsening of fibrosis Liver death	A	Vitamin D serum levels	Wang *et al.*,[25] Ahn *et al.*,[22] Lu *et al.*,[23] Batai *et al.*,[46]
	rs222020	T	Worsening of fibrosis	T/C	Vitamin D serum levels	Porter *et al.*[28] Xu *et al.*,[49] Bu *et al.*,[27] Zhang *et al.*,[31]
CYP2R1	rs7116978	T	Ascites	C	Vitamin D serum levels	Nissen *et al.*,[29]
	rs1562902	C	HCC	C/T	Vitamin D serum levels	Wjst *et al.*,[30] Bu *et al.*,[27] Zhang *et al.*,[31] Nissen *et al.*,[29]
VDR	rs4516035	C	Gastric/esophageal bleeding	C	Liver fibrosis	Beilfuss *et al.*,[8]
	rs2239186	G	HCC	G/A	Vitamin D serum levels	Wjst *et al.*,[30] Yao *et al.*,[32]
SMAD3	rs2118610	T/C	Worsening of fibrosis Hepatic encephalopathy	C	Skin scarring	Brown *et al.*,[33]
TGFB1	rs1800469[a]	A	Hepatic decompensation CTP>7	A	*HCC and cirrhosis*	Radwan *et al.*,[37] Mohy *et al.*[35] Ma *et al.*,[36]

[a]Polymorphism associated after Bonferroni's correction.

related to both risk of and protection from low levels of vitamin D.[27,30,31] We described various SNPs in *DHCR7*, *GC* and CYP2R1 genes with tendency towards significance with the studied outcomes, however our results were controversial or did not corroborate the literature.

The interaction between VDR, TGFβ-1 and SMAD3 needs to be considered more directly in the context of liver fibrosis and the progression of liver disease following it. Though we only found polymorphisms with tendency towards significance in *VDR*, those findings are in accordance to which is described in the literature. The C allele of rs4516035, related in this sample to gastric/esophageal bleeding, was described to be related to an increased expression of TGFβ-1 and α-SMA mRNA in the liver of patients with non-alcoholic fatty liver disease.[8] Also, the G allele of rs2239186, related to HCC in this sample, has been described to be linked to both lower and higher levels of vitamin D.[30,32] We expected to find an association between SNPs at the *VDR* locus and worsening fibrosis in this cohort; however, none of the SNPs in the *VDR* gene were significant for liver fibrosis, and neither was the bAt [CCA] haplotype. This may be due to the fact that the differences in CCA haplotype frequencies found by Baur *et al.*[10] were caused mainly by rs7975232, which was not significant in our sample. In our study, *SMAD3* gene demonstrated controversial findings; T allele of rs2118610 tended towards significance with progression of liver fibrosis, and C allele to presence of hepatic encephalopathy. Although we have not found any association of this polymorphism with liver disease in the literature, Brown *et al.*[33] found that the C allele might play a role in keloid formation in Afro-Caribbeans. In this cohort, the AA genotype of rs1800469 in the *TGFB1* gene was the only one with statistical association after Bonferroni's correction, being associated with hepatic decompensation. Transforming growth factor beta plays a central role in many fibrotic diseases, including chronic hepatitis, and TGF-β signaling in these processes has been attributed to a canonical pathway involving Smad proteins.[34] The A allele of rs1800469 has been linked to both increased levels of TGF-β1 and risk of cirrhosis,[35] as well as related to the development of HCC in Chinese and Egyptian patients.[36,37] Therefore, we believe our results and the ones reported in Chinese and Egyptians populations lean toward the same direction, since HCC is the natural evolution of cirrhosis. Maybe a follow-up period of four years may not be sufficient to HCC development. On the other hand, these associations may be population-specific and it is possible that Egyptians and Chinese are not comparable to the American population analyzed in the HALT-C Cohort. We should keep in mind that the association found here between rs1800469 and hepatic decompensation may be only a result of the severity of cirrhosis rather than being related to vitamin D pathway itself.

Low levels of vitamin D have been linked to increased mortality among cirrhotic patients.[38] None of the studied polymorphisms were related to liver transplant and/or death. *VDR* polymorphisms have been associated to acute cellular rejection in liver-transplanted patients.[39] However, there was no information on post-transplant events among the available clinical HALT-C data.

Our study has potential limitations. Vitamin D serum levels were not available to confirm the relationship between polymorphisms and variants. Corey *et al.*[40] published an ancillary study on vitamin D serum levels in 129 cases and 129 controls of the HALT-C cohort. They defined as cases those patients who experienced an increase of at least 2 points in their Ishak fibrosis score or who developed hepatic decompensation over the course of the study. The controls were subjects with stage 3 or 4 fibrosis who did not reach any of these endpoints. In comparative analyses, mean 25-OH VD measures did not differ between groups. This shows that analyses of associations between punctual 25-OH VD serum levels and hard outcomes can be misleading, since 25-OH VD serum levels fluctuate as a consequence of seasons, age, race, gender, among other factors.

The original HALT-C study tested whether PEG-INF treatment had an effect on disease progression. The participants' treatment status could represent a potential confounder for the present study, and we did not have access to this information. However, since the HALT-C study did not find any association between PEG-INF treatment and disease progression, we believe that our results are robust.[41] Also,no information regarding viral factos were available, such as viral load and genotype. However we do know that the great majority of enrolled patients were genotype 1, as described by Di Bisceglie.[41]

Even though the existence of other polymorphisms, either in the genes studied here or in other genes related to vitamin D metabolism, cannot be ruled out, we consider it a strength of our study that we included only the SNPs most likely to be associated with vitamin D metabolism in liver disease progression. We view our results as exploratory, pending further confirmation on an independent cohort to validate our findings. We decided to present SNPs reaching a 0.05 threshold, since corrections for multiple testing could be too stringent, risking the suppression of interesting findings.[42–44] However, multiple measurements may incur a risk for type I errors and these results should be looked at with care. Of the 40 analyzed SNPs, eleven presented tendency towards significance, reaching a 0.05 threshold, while only rs1800469 in *TGFB1* was significant after Bonferroni correction. Also, it is worth noting that the effective sample size was relatively small for all outcomes (Figure 2). It is well established that the sample size is one of the most important factors affecting the power of genetic association studies for complex phenotypes.[45] The fact that weak association signals could be detected in this relatively small sample therefore suggests that these SNPs are worth investigating in larger cohorts.

In conclusion, polymorphisms underlying vitamin D metabolism and effects showed a possible association with important outcomes in chronic hepatitis C. Here, we reported eleven polymorphisms that must be confirmed in a larger cohort and that could potentially be used in further studies to evaluate the role of vitamin D in chronic hepatitis C; and one rs1800469 in *TGFB1* gene that was associated to hepatic decompensation in these patients.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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