

DEPDC5 Variants Increase Fibrosis Progression in Europeans With Chronic Hepatitis C Virus Infection

Maria Antonella Burza,^{1*} Benedetta Maria Motta,^{1*} Rosellina Margherita Mancina,¹ Piero Pingitore,¹ Carlo Pirazzi,¹ Saverio Massimo Lepore,² Rocco Spagnuolo,³ Patrizia Doldo,³ Cristina Russo,² Veronica Lazzaro,² Janett Fischer,⁴ Thomas Berg,⁴ Alessio Aghemo,⁵ Cristina Cheroni,⁶ Raffaele De Francesco,⁶ Silvia Fargion,⁷ Massimo Colombo,^{5,7} Christian Datz,⁸ Felix Stickel,⁹ Luca Valenti,⁷ and Stefano Romeo^{1,2,10}

Chronic hepatitis C virus (HCV) infection may progress to cirrhosis and hepatocellular carcinoma (HCC). Recently, two genetic variants, *DEPDC5* rs1012068 and *MICA* rs2596542, were associated with the onset of HCC in Asian subjects with chronic HCV infection. The aim of the present study was to analyze whether *DEPDC5* and *MICA* genetic variants were associated with liver disease progression in European subjects with chronic HCV infection. In a Northern Italian discovery cohort (n = 477), neither *DEPDC5* rs1012068 nor *MICA* rs2596542 were associated with HCC (n = 150). However, *DEPDC5* rs1012068 was independently associated with cirrhosis (n = 300; *P* = 0.049). The association of rs1012068 with moderate to severe fibrosis was confirmed in an independent cross-sectional German cohort (n = 415; *P* = 0.006). Furthermore, *DEPDC5* rs1012068 predicted faster fibrosis progression in a prospective cohort (n = 247; *P* = 0.027). Next, we examined the distribution of nonsynonymous *DEPDC5* variants in the overall cross-sectional cohort (n = 912). The presence of at least one variant increased the risk of moderate/severe fibrosis by 54% (*P* = 0.040). To understand the molecular mechanism underlying the genetic association of *DEPDC5* variants with fibrosis progression, we performed *in vitro* studies on immortalized hepatic stellate cells (LX-2). In these cells, down-regulation of *DEPDC5* resulted in increased expression of β -catenin and production of its target matrix metalloproteinase 2 (MMP2), a secreted enzyme involved in fibrosis progression. **Conclusion:** *DEPDC5* variants increase fibrosis progression in European subjects with chronic HCV infection. Our findings suggest that *DEPDC5* down-regulation may contribute to HCV-related fibrosis by increasing MMP2 synthesis through the β -catenin pathway. (HEPATOLOGY 2016;63:418-427)

Chronic hepatitis C virus (HCV) infection is an important health issue affecting approximately 3% of the population worldwide.¹ Although many subjects show only mild or no symp-

toms in the early stages, chronic HCV infection frequently leads to cirrhosis and its complications, including hepatocellular carcinoma (HCC).²⁻⁴ The progression to cirrhosis and HCC is influenced by

Abbreviations: CI, confidence interval; DEPDC5, DEP domain-containing 5; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; MICA, MHC class I polypeptide-related sequence A; MMP2, matrix metalloproteinase 2; OR, odds ratio; SMA, smooth muscle actin; TIMP1, tissue inhibitor of metalloproteinase 1; TIMP2, tissue inhibitor of metalloproteinase 2.

From the ¹Department of Molecular and Clinical Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden; ²Clinical Nutrition Unit, Department of Medical and Surgical Sciences, Magna Graecia University, Catanzaro, Italy; ³Department of Experimental and Clinical Medicine, Magna Graecia University, Catanzaro, Italy; ⁴Department of Gastroenterology and Rheumatology, Section of Hepatology, University Hospital, Leipzig, Germany; ⁵Department of Gastroenterology, Fondazione IRCCS Ca' Granda Ospedale Policlinico Milano, Milan, Italy; ⁶Virology Program, INGM-Istituto Nazionale di Genetica Molecolare "Romeo ed Enrica Invernizzi", Milan, Italy; ⁷Università degli Studi di Milano, Fondazione IRCCS Ca'Granda Ospedale Policlinico Milano, Department of Pathophysiology and Transplantation, Milan, Italy; ⁸Department of Internal Medicine, Hospital Oberndorf, Teaching Hospital of the Paracelsus Private University of Salzburg, Oberndorf, Austria; ⁹Department of Gastroenterology and Hepatology, University Hospital of Zürich, Rämistrasse 100, CH-8091, Zürich, Switzerland; ¹⁰Department of Cardiology, Sahlgrenska University Hospital, Gothenburg, Sweden

*These authors contributed equally to this work.

Received March 5, 2015; accepted October 25, 2015.

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.28322/supinfo.

both environmental (e.g., age, coinfection, alcohol)⁵⁻⁷ and genetic factors.⁸⁻¹³

To date, two studies have examined the susceptibility to HCC in subjects with chronic HCV infection at a genome-wide level.^{14,15} Those two genome-wide association studies found that noncoding genetic variants in the DEP domain-containing 5 (*DEPDC5*)¹⁴ and in the MHC class I polypeptide-related sequence A (*MICA*)¹⁵ loci confer susceptibility to HCC in Asian subjects with chronic HCV infection. However, ethnicity influences the effect of genetic variants.¹⁶ Allele frequencies and haplotype patterns vary considerably, and the effect size of polymorphisms changes across different populations.¹⁷ Thus, it is important to study whether the effect of genetic variants may be applied to other populations.

The aim of the study was to examine the effect of *DEPDC5* rs1012068 (T>G), and *MICA* rs2596542 (C>T) variants on HCC onset in Europeans with chronic HCV infection.

Subjects and Methods

Study Design. We tested our hypotheses in a discovery cohort (n = 477) and, subsequently, in independent validation cohorts, which comprised a cross-sectional cohort (n = 415) and a prospective cohort (n = 247). Both the discovery and the validation cohorts consisted of therapy-naïve subjects with chronic HCV infection and no HBV or HIV coinfection. Only the significant associations were carried on in the validation cohorts. Figure 1 illustrates the study design and the flow of the analyses. To analyze the association between genetic variants and HCC, we included only patients with cirrhosis, because cirrhosis is the main risk factor

for HCC.¹⁸ To test the association with fibrosis and maximize the power of our analysis, we compared the genotype distribution of the *DEPDC5* rs1012068 and *MICA* rs2596542 variants in the two extremes of fibrosis distribution in the discovery cohort: subjects with no/mild fibrosis (stage F0-F1) versus those with cirrhosis (i.e., the most severe stage, F4). Next, we validated the association found with cirrhosis in the cross-sectional validation cohort and extended it to the full spectrum of fibrosis. Finally, to confirm our findings in a prospective way, we analyzed the association between *DEPDC5* rs1012068 and fibrosis progression rate (FPR) in the prospective validation cohort. The analysis of nonsynonymous variants was performed by pooling together the discovery and the cross-sectional validation cohorts.

Discovery Cohort. The discovery cohort consisted of 477 subjects with chronic HCV infection enrolled at Fondazione IRCCS Ca' Granda Milan, Italy. Subjects were selected according to the criteria defined above in the study design from a larger cohort that has been described previously.¹⁹ The degree of liver fibrosis was assessed histologically according to the METAVIR²⁰ classification. We performed two different analyses by using two different subsets of this cohort. First, to test the association between *DEPDC5* rs1012068, *MICA* rs2596542, and HCC, we analyzed only those subjects with cirrhosis (n = 150 with and n = 150 without HCC). Next, we examined subjects with no/mild fibrosis and those with cirrhosis. No/mild fibrosis was defined as stage F0-F1 and cirrhosis as stage F4. All subjects gave informed consent to participate in the study, which was approved by the local ethics committee. The demographic and clinical characteristics of the discovery cohort are given in Table 1.

S.R. was supported by the Swedish Research Council (VR, 254439006), the Swedish Heart Lung Foundation (244439007), the Swedish federal government funding under the LUA/ALF agreement (76290), and the Novo Nordisk Foundation Grant for Excellence in Endocrinology (244439012). L.V. was supported by Ricerca Corrente Fondazione Ca'Granda IRCCS Policlinico of Milan, Associazione Malattie Metaboliche del Fegato ONLUS, and Bando Medicina Molecolare Fondazione IRCCS Ca'Granda-Istituto Nazionale Genetica Molecolare 2015-2016. R.M.M. was supported by Nilsson-Ehle funds from the Fysiografiska Sällskapet in Lund, Sweden. S.M.L. and V.L. were supported by a cofinanced grant from the European Commission, the European Social Fund, and the Calabria Region. J.F. and T.B. were supported by the German Competence Network for Viral Hepatitis (Hep-Net), funded by the German Ministry of Education and Research (BMBF) (grant no. 01 KI 0437, project no. 10.1.3, and core project no. 10.1 [Genetic Host Factors in Viral Hepatitis and Genetic Epidemiology Group in Viral Hepatitis]); the EU-Vigilance Network of Excellence Combating Viral Resistance (VIRGIL) (project no. LSHM-CT-2004-503359); and the BMBF Project: Host and Viral Determinants for Susceptibility and Resistance to Hepatitis C Virus Infection (grant no. 01KI0787). F.S. was supported by unrestricted research support from AbbVie AG (Switzerland) and Gilead (Switzerland) and additional funding from the Swiss National Funds (grant no. 310030_138747).

Address reprint requests to: Stefano Romeo, M.D., Ph.D., Department of Molecular and Clinical Medicine, The Wallenberg Laboratory, Bruna Stråket 16, Gothenburg 413 34, Sweden. E-mail: stefano.romeo@wlab.gu.se. Address reprint requests to: Luca Valenti, M.D., Ph.D., Department of Pathophysiology and Transplantation, University of Milan, Fondazione IRCCS Ca' Granda Ospedale Policlinico Pad. Granelli, Via Sforza 35, 20122 Milan, Italy. E-mail: luca.valenti@unimi.it.

Copyright © 2015 The Authors. HEPATOLOGY published by Wiley Periodicals, Inc., on behalf of the American Association for the Study of Liver Diseases. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is noncommercial and no modifications or adaptations are made.

View this article online at wileyonlinelibrary.com.

DOI 10.1002/hep.28322

Potential conflict of interest: Felix Stickel has received grants from Gilead and AbbVie and is a consultant for BioTech.

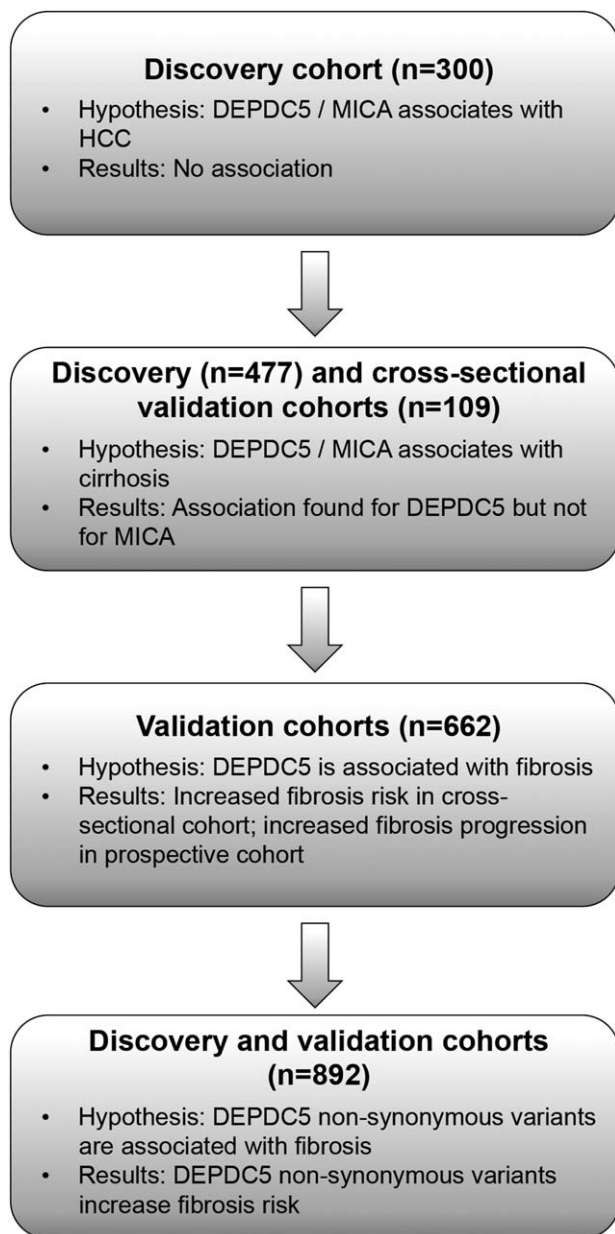


Fig. 1. Study design and flow of the analyses. This picture illustrates the study design and flow of analyses. Both the discovery and the validation cohorts consist of therapy-naïve subjects with chronic HCV infection. The discovery cohort consisted of a cross-sectional group of 477 subjects enrolled in Milan (Italy). In the analysis on HCC, only patients with cirrhosis were included. To test the association with fibrosis, we first analyzed the extremes of fibrosis distribution (stage F0-F1 versus F4) to maximize the power by selecting two extremely different groups. Next, we examined the association with the full spectrum of fibrosis in the cross-sectional validation cohort (the Leipzig and Bern cohorts pooled together [n = 415]) and with fibrosis progression in the prospective cohort (Milan prospective cohort [n = 247]). Only the significant associations were carried on in the validation cohorts. The analysis of nonsynonymous variants was performed by pooling together the discovery and the cross-sectional validation cohorts.

Validation Cohorts. The validation cohorts consisted of subjects with chronic HCV infection and included a cross-sectional cohort (n = 415) and a pro-

spective cohort (n = 247). The cross-sectional cohort was created by pooling together a cohort from Bern, Switzerland, and a cohort from Leipzig, Germany, the latter of which has been described previously.²¹ The current report includes 268 subjects with chronic HCV infection and the full spectrum of liver disease. The Bern cohort included 147 therapy-naïve patients with chronic HCV from Switzerland. The stage of liver fibrosis was histologically assessed according to the META-VIR²⁰ classification. The prospective cohort comprised 247 subjects that were enrolled at the University of Milan in Milan, Italy. The characteristics of this cohort have been described in detail previously.^{22,23} The FPR was calculated as the ratio between the Ishak score²⁴ and the disease duration (years) and was considered a continuous variable. The mean disease duration was 25 ± 10 years; log₁₀ transformation of FPR was used to obtain linearity. Ethical approval to include patients for validation was obtained from all participating institutions, and all patients signed informed consent to be included in a genetic analysis. The demographic and clinical characteristics of each discovery cohort are given in Table 1.

Selection of DEPDC5 Nonsynonymous Variants. By using the Ensembl genome browser,²⁵ we selected all the *DEPDC5* nonsynonymous variants with a minor allele frequency $\geq 1\%$ in Europeans according to 1000 Genomes Project data. We specifically focused on the nonsynonymous variants because they result in an amino acidic substitution and likely affect the protein function. Linkage disequilibrium estimations for the *DEPDC5* variants were examined from the Ensembl

Table 1. Demographic and Clinical Characteristics of the Cohorts

| | Discovery Cohort | | Validation Cohort | |
|----------------------|-----------------------|----------------------|-------------------|-------------------|
| | Milan | Leipzig | Bern | Milan Prospective |
| N | 477 | 268 | 147 | 247 |
| Age, years | 63 \pm 12 | 52 \pm 12 | 48 \pm 11 | 47 \pm 11 |
| Sex | | | | |
| Men | 317 (67) | 130 (49) | 93 (63) | 129 (52) |
| Women | 160 (33) | 138 (51) | 54 (37) | 118 (48) |
| Alcohol consumption | | | | |
| None/mild* | 433 (91) | 108 (77) | 110 (75) | 247 (100) |
| At-risk [†] | 44 (9) | 32 (23) [‡] | 37 (25) | 0 (0) |
| Liver fibrosis stage | | | | |
| F0-F1 | 177 (37) | 105 (39) | 68 (46) | 157 (64) |
| F2-F4 | 300 (63) [§] | 163 (61) | 79 (54) | 90 (36) |
| Cirrhosis | 300 (63) | 72 (27) | 37 (25) | — |
| HCC | 150 (31) | 50 (19) | 8 (5) | 0 (0) |

Data are presented as the mean \pm standard deviation or as n (%).

*Defined as 0-20 g/day for women and 30 g/day for men.

[†]Defined as >20 g/day for women and >30 g/day for men.

[‡]Data on alcohol intake were not available in 128 subjects (48%).

[§]All subjects in this cohort had stage F4 fibrosis.

genome browser data.²⁵ Detailed information on these variants is reported in [Supporting Table 1](#).

Genotyping of Genetic Variants. The fluorogenic 5'-nucleotidase allelic discrimination assay (TaqMan Applied Biosystems, Foster City, CA) was used to genotype all the *DEPDC5* variants and *MICA* rs2596542 in the discovery and the cross-sectional cohorts according to the manufacturer's instructions, with a $\geq 95\%$ success rate. In the prospective validation cohort, *DEPDC5* rs1012068 genotyping was performed by Human660W-Quad BeadChip (Illumina, San Diego, CA), followed by single-nucleotide polymorphism calling using the default settings of Genome Studio software. All genotype distributions were in Hardy-Weinberg equilibrium.

Gene Expression. *DEPDC5* messenger RNA (mRNA) expression was assessed in seven immortalized human cell lines. To assess mRNA expression in cultured cells, total RNA was isolated from each cell type using an RNeasy Mini Kit (Qiagen, Valencia, CA). First-strand complementary DNAs were synthesized from 1 μ g RNA using a reverse-transcription kit (Applied Biosystems). *DEPDC5* and β -catenin mRNA expression were assessed in LX-2 control cells and in LX-2 *DEPDC5* knockout cells. TaqMan probe and master mix (Life Technologies, Carlsbad, CA, USA) were used in a total volume of 20 μ L per reaction. Real-time polymerase chain reaction (PCR) assay was performed on a CFX Real-Time PCR Detection System (Bio-Rad, Hercules, CA). *DEPDC5* expression levels were assessed using the delta-delta C_t method and normalization to β -actin.

DEPDC5 Knockdown. LX-2 cells were transfected with *DEPDC5* small interfering RNA (siRNA) or negative control (scramble) siRNA (Ambion-Life Technologies by Thermo Fisher Scientific, Rockford, IL) with TurboFect Transfection Reagent according to the manufacturer's instructions. The cells were transfected with 25 nM siRNA oligos for 48 hours in serum-free medium. RNA was isolated from each cell type using an RNeasy Mini Kit (Qiagen). First-strand complementary DNAs were synthesized from 1 μ g RNA using a reverse-transcription kit (Applied Biosystems) to confirm *DEPDC5* knockdown.

Cell Proliferation. LX-2 cells were seeded in six-well plates. When 50% confluent, cells were transfected with *DEPDC5* siRNA or scramble siRNA. Cell numbers were counted 24, 48, and 72 hours after transfection using the Nucleocounter NC-100 (Chemometec A/S, Lillerød, Denmark).

Immunoblot Analysis. LX-2 cells were seeded in T-75 plates. When 50% confluent, cells were transfected with *DEPDC5* siRNA or scramble siRNA. Cells were collected 48 hours after transfection and were lysed in

M-PER Mammalian Protein Extraction Reagent (Pierce, Thermo Fisher Scientific) containing complete protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Immunoblot analysis was performed according to standard procedures. Bands were visualized using a Chemidoc XRS System and Image Lab Software (Bio-Rad).

The following antibodies were used: rabbit anti-collagen I (Sigma-Aldrich), rabbit anti-matrix metalloproteinase 2 (MMP2) (Sigma-Aldrich), mouse anti- α -smooth muscle actin (SMA) (Sigma-Aldrich), rabbit anti-tissue inhibitor of metalloproteinase 1 (TIMP1) (Abcam, Cambridge, UK), mouse anti-tissue inhibitor of metalloproteinase 2 (TIMP2) (Sigma-Aldrich), and rabbit anti-Calnexin (Abcam).

Statistical Analyses. Data are shown as the mean and standard deviation or number and proportion. The distribution of *DEPDC5* rs1012068 and *MICA* rs2596542 genotypes between the different groups was compared using Fisher's exact test. In the analysis of nonsynonymous variants, we compared the proportion of subjects with at least one nonsynonymous allele between subjects with no/mild fibrosis and those with moderate/severe fibrosis by Fisher's exact test. Binary logistic regression was used to evaluate the effect of the *DEPDC5* rs1012068 on severe fibrosis including other risk factors in the model. The association between *DEPDC5* rs1012068 and FPR was performed by generalized linear model adjusted by age at liver biopsy and sex. Analyses were performed using IBM SPSS Statistics, Version 20.0 (IBM, Armonk, NY). *DEPDC5* and β -catenin expressions between LX-2 control cells and LX-2 *DEPDC5* knockout cells were compared using an independent samples Mann-Whitney U test.

Results

***DEPDC5* rs1012068 and *MICA* rs2596542 Are Not Associated With HCC in Europeans With Chronic HCV Infection.** To examine the association of *DEPDC5* rs1012068 and *MICA* rs2596542 with HCC in Europeans, we tested whether the genotype distribution of these variants was different between patients with cirrhosis who did or did not also have HCC from the discovery cohort ($n = 300$). We specifically analyzed only patients with cirrhosis because cirrhosis is the main risk factor for HCC. We observed no difference in the *DEPDC5* rs1012068 and *MICA* rs2596542 distribution between subjects with HCC and those with cirrhosis ([Supporting Table 2](#)). To increase the power of the analysis, we pooled together the discovery and the cross-sectional validation cohorts ($n = 403$). No difference in *DEPDC5* rs1012068 and *MICA* rs2596542 distribution

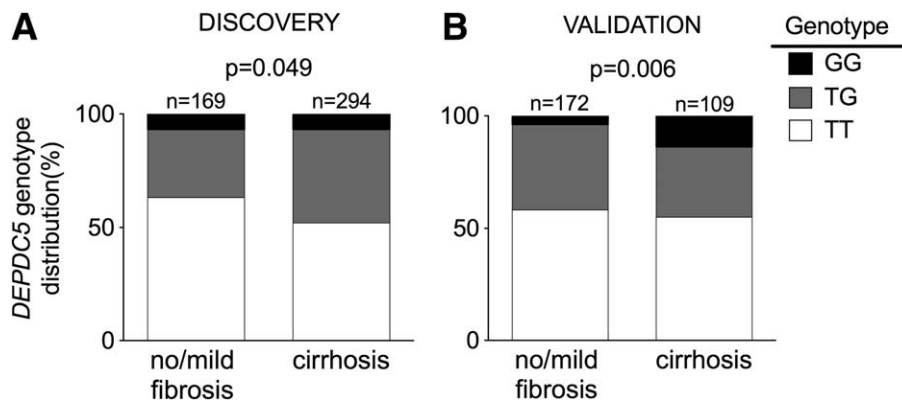


Fig. 2. *DEPDC5* rs1012068 is associated with cirrhosis in European subjects with chronic HCV infection. (A) Genotype distribution of *DEPDC5* rs1012068 in the discovery cohort. (B) Genotype distribution of *DEPDC5* rs1012068 in the cross-sectional validation cohort. The validation cohort consisted of subjects from the Leipzig and Bern cohorts pooled together. Carriers of the G allele also had a higher risk of cirrhosis (OR, 1.46; 95% CI, 1.04-2.04; $P = 0.027$ after pooling together the discovery and the validation cohorts and adjusting for age, sex, and recruitment center). The exact frequencies are shown in Supporting Table 3.

was observed between subjects with HCC and those with cirrhosis in the pooled cohort (Supporting Table 2).

DEPDC5 rs1012068, But Not MICA rs2596542, Is Associated With Cirrhosis. Based on the lack of association between *DEPDC5* rs1012068 and *MICA* rs2596542 with HCC, we hypothesized that these two genetic variants might be associated with fibrosis rather than HCC. To test this hypothesis and increase the power to detect the association, we analyzed the association between *DEPDC5* rs1012068 and *MICA* rs2596542 and fibrosis by analyzing the extreme stages of fibrosis in the discovery cohort. Particularly, we compared the genotype distribution of these genetic variants between subjects with no/mild fibrosis and those with cirrhosis. Thus, based on the fibrosis grade defined histologically, we stratified the discovery cohort in subjects with no/mild fibrosis ($n = 177$; stage F0-F1) and those with cirrhosis ($n = 300$; stage F4). The prevalence of *DEPDC5* rs1012068 was higher in subjects with cirrhosis than in those with no/mild fibrosis ($P = 0.049$; Fig. 2A and Supporting Table 3). To confirm the association between *DEPDC5* rs1012068 and cirrhosis, we analyzed the cross-sectional validation cohort. We found that the prevalence of *DEPDC5* rs1012068 was higher in subjects with cirrhosis (F4) than in those with no/mild (F0-F1) fibrosis ($P = 0.006$; Fig. 2B and Supporting Table 3). The *DEPDC5* rs1012068 G allele was associated with a 40% increased risk of cirrhosis (odds ratio [OR], 1.40; 95% confidence interval [CI], 1.08-1.81; $P = 0.011$; Supplementary Table 4) after adjusting for age, sex, and recruitment center in the discovery and validation cohorts.

On the contrary, the distribution of *MICA* rs2596542 genotypes was not significantly different according to the presence or absence of cirrhosis ($P = 0.075$; Supporting Table 5).

The DEPDC5 rs1012068 Genetic Variant Is Associated With Fibrosis Severity in the Validation Cohorts.

To confirm our hypothesis that *DEPDC5* rs1012068 variant is associated not only with cirrhosis but, in general, with a more severe fibrosis, we examined the cross-sectional validation cohort including subjects with the entire spectrum of fibrosis. To confirm the association with fibrosis progression, we next analyzed the prospective validation cohort where the fibrosis progression rate has been assessed.

First, we examined the cross-sectional cohort and stratified it in subjects with no/mild fibrosis ($n = 173$; stage F0-F1) and those with moderate/severe fibrosis ($n = 242$; stage F2-F4). Consistent with the findings on cirrhosis, the prevalence of *DEPDC5* rs1012068 was higher in subjects with moderate/severe fibrosis than in those with no/mild fibrosis ($P = 0.006$; Fig. 3A and Supporting Table 3). *DEPDC5* rs1012068 variant was an independent risk factor for moderate/severe fibrosis (OR, 1.52; 95% CI, 1.09-2.12; $P = 0.015$ per each G allele; Supporting Table 6) after adjusting for age, sex, and recruitment center. Results were virtually identical after adjusting for diabetes and at-risk alcohol intake (Supporting Table 6).

Next, we tested whether *DEPDC5* rs1012068 was associated with higher FPR in the prospective cohort. Carriers of this variant had a greater FPR (adjusted $P = 0.027$; Fig. 3B and Table 2), independently of age at liver biopsy and sex.

DEPDC5 Nonsynonymous Variants Confer Susceptibility to Liver Fibrosis.

We next investigated whether the *DEPDC5* gene locus is directly associated with the development of moderate/severe liver fibrosis. We examined the distribution of and risk conferred by *DEPDC5* nonsynonymous variants at the onset of

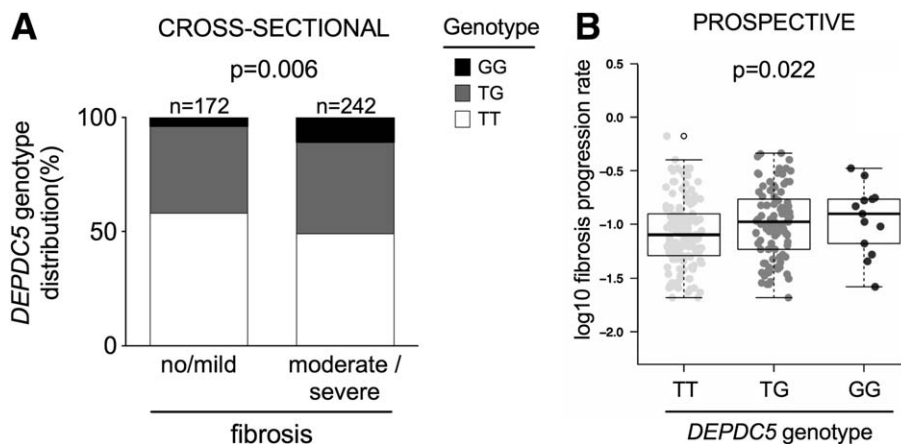


Fig. 3. *DEPDC5* rs1012068 is associated with fibrosis progression in Europeans with chronic HCV infection. The genotype distribution of *DEPDC5* rs1012068 is shown in the cross-sectional (A) and in the prospective (B) validation cohorts. The cross-sectional validation cohort consisted of subjects from Leipzig and Bern cohorts pooled together. Carriers of the G allele also had a higher risk of moderate/severe fibrosis (OR, 1.46; 95% CI, 1.04-2.04; $P = 0.027$ after adjusting for age, sex, and recruitment center). The association between *DEPDC5* rs1012068 and FPR was calculated by multivariate generalized linear models under an additive genetic model (see Table 2 for further details).

moderate/severe fibrosis. The genotype distribution of the *DEPDC5* nonsynonymous variants is shown in Supporting Table 7. To increase the power of our analysis, we analyzed the discovery and the cross-sectional validation cohorts pooled together. Specifically, we compared the presence of *DEPDC5* nonsynonymous variants between subjects with no/mild fibrosis (stage F0-F1) and those with moderate/severe fibrosis (stage F2-F4). Subjects with severe fibrosis had a higher, albeit non-significant ($P = 0.097$) prevalence of at least one *DEPDC5* nonsynonymous variants than those with no/mild fibrosis (67% versus 60%, respectively).

Our analysis of the risk of developing moderate/severe fibrosis revealed that carriage of at least one nonsynonymous variant was associated with a 54% increase of the risk of moderate/severe fibrosis (OR, 1.54; 95% CI, 1.02-2.34; $P = 0.040$; Table 3).

DEPDC5 Is Highly Expressed in Immortalized Hepatic Stellate Cells and Its Knockout Increases β -catenin Expression and MMP2 Production.

DEPDC5 has a DEP domain involved in several cellular functions, including the Wnt/ β -catenin pathway.^{26,27} The Wnt/ β -catenin pathway is involved in liver fibro-

genesis by affecting hepatic stellate cells activation.^{28,29} Therefore, to test the hypothesis that *DEPDC5* increases fibrosis through this pathway, we first assessed *DEPDC5* expression in seven different human immortalized cell types. We found that *DEPDC5* is highly expressed in LX-2 cells, a human hepatic stellate cell line spontaneously immortalized through culture in low serum³⁰ (Fig. 4A). Next, to test whether *DEPDC5* is involved in hepatic stellate cell activation through the Wnt/ β -catenin pathway, we incubated LX-2 cells with and without *DEPDC5* siRNA (Ambion) for 48 hours. *DEPDC5* knockdown induces an increase in β -catenin expression in LX-2 cells ($P = 0.002$; Fig. 4B).

Next, we tested the effect of *DEPDC5* knockdown on cell proliferation. Cell numbers were counted 24, 48, and 72 hours after transfection, and we did not observe any increase on LX-2 cell proliferation after *DEPDC5* knockdown (Supporting Fig. 1).

Furthermore, we tested whether *DEPDC5* knockdown affects the synthesis of molecules involved in liver fibrogenesis, namely collagen, MMP2, α -SMA, TIMP1, and TIMP2.^{30,31} *DEPDC5* knockdown resulted in an increase in MMP2 production ($P < 0.05$; Fig. 4C,D) and in no differences in collagen, α -SMA, TIMP1, and TIMP2 production (Fig. 4C,D).

Table 2. *DEPDC5* rs1012068 Is Associated With Higher Fibrosis Progression Rate in European Subjects With Chronic HCV Infection

| | Estimate* | Standard Error | P | Estimate† | Standard Error | P |
|---------------------------|-----------|----------------|-------|-----------|----------------|-------|
| <i>DEPDC5</i> , G alleles | 0.078 | 0.034 | 0.022 | 0.074 | 0.033 | 0.027 |
| Age at biopsy, years | 0.001 | 0.001 | 0.54 | 0.002 | 0.002 | 0.31 |
| Sex, men | 0.045 | 0.020 | 0.026 | 0.047 | 0.020 | 0.020 |

*Unadjusted.

†Adjusted for age at biopsy and sex.

Table 3. Carriers of *DEPDC5* Nonsynonymous Variants Have Higher Risk of Moderate/Severe Fibrosis

| | OR | 95% CI | P |
|------------------------|------|-----------|--------|
| Age, years | 1.05 | 1.04-1.07 | <0.001 |
| Sex, men | 1.33 | 0.98-1.81 | 0.066 |
| Recruitment center | 0.88 | 0.76-1.02 | 0.095 |
| Nonsynonymous variants | 1.54 | 1.02-2.34 | 0.040 |

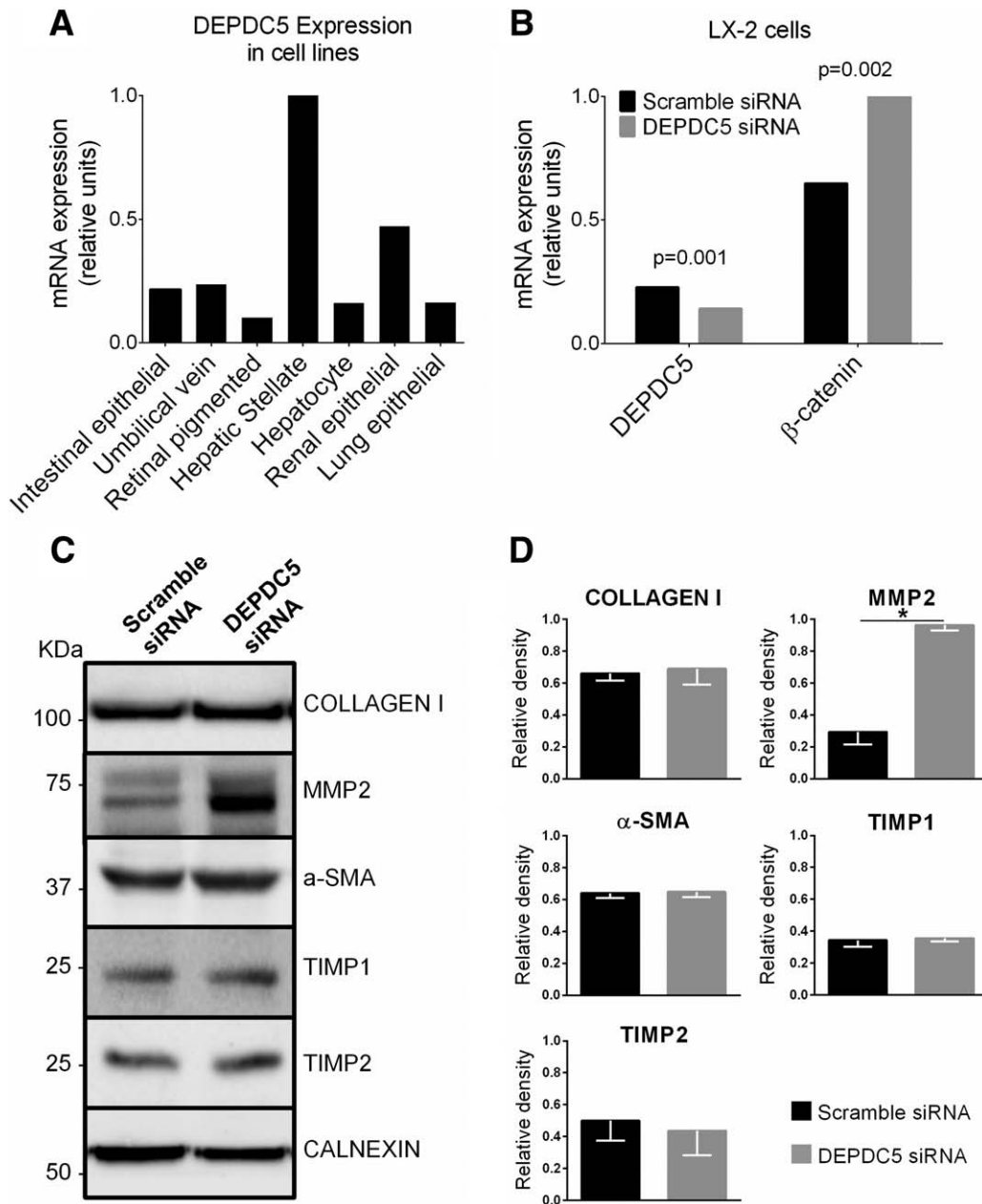


Fig. 4. *DEPDC5* is highly expressed in LX-2 cells and *DEPDC5* silencing induces β -catenin levels and MMP2 production in LX-2 cells. (A) *DEPDC5* mRNA expression in human tissues assessed by quantitative PCR. The tissue with the highest C_t value was assigned a value of 1. (B) β -catenin and *DEPDC5* mRNA expression in LX-2 cells incubated with negative control (scramble) siRNA or *DEPDC5* siRNA for 48 hours. The experiments were repeated four times. *DEPDC5* reduction ($P = 0.001$) and β -catenin increase ($P = 0.002$) were significant. (C) Lysate fractions of LX-2 cells transfected with scramble siRNA or *DEPDC5* siRNA for 48 hours analyzed by western blotting. Calnexin was used as loading control. (D) Quantified relative protein levels are shown as column charts. The data are the means \pm standard deviation of four independent experiments ($P < 0.05$).

Discussion

In this study, we examined the association between severe liver disease and *DEPDC5* rs1012068 in European subjects with chronic HCV infection. We showed that *DEPDC5* variants confer susceptibility to severe fibrosis.

Genome-wide association studies identified *DEPDC5* rs1012068 and *MICA* rs2596542 as loci of susceptibil-

ity to HCC in Asian subjects with chronic HCV infection.^{14,15} In the current study, we examined whether genotype distribution was different in European subjects with cirrhosis who did and did not also have HCC. We examined those two specific groups because HCC occurs primarily in patients with cirrhosis.¹⁸ We found that neither *DEPDC5* rs1012068 nor *MICA* rs2596542 are associated with HCC in Europeans with chronic

HCV infection. To understand the discrepancy between our results and those reported in Asian subjects,^{14,15} we analyzed the association between those two variants and liver fibrosis. To maximize the power of our analysis, we compared the genotype distribution of the *DEPDC5* rs1012068 and *MICA* rs2596542 variants in the two extremes of fibrosis distribution: no/mild fibrosis (F0-F1) versus the most severe stage of fibrosis (F4 [i.e., cirrhosis]). We found that *MICA* rs2596542 was not associated with cirrhosis, but we observed an enrichment of *DEPDC5* rs1012068 in subjects with cirrhosis. This enrichment was confirmed in an independent cross-sectional validation cohort.

To confirm our hypothesis that the *DEPDC5* rs1012068 variant is associated with, in general, a more severe fibrosis, we examined two validation cohorts: the cross-sectional and prospective cohorts, which included subjects with the entire spectrum of fibrosis. In the cross-sectional cohort, *DEPDC5* rs1012068 conferred an approximately 50% increased risk of fibrosis independently of age and sex. This association was confirmed in the prospective cohort by observing that carriers of *DEPDC5* rs1012068 had a higher fibrosis progression rate. Taken together, our data suggest that *DEPDC5* rs1012068 confers susceptibility to the development of severe fibrosis, rather than HCC, in European subjects with chronic HCV infection.

The apparent discrepancy between our results and those reported by the two genome-wide association studies in Asian subjects^{14,15} might be due to a key difference in the study design, as also pointed out by Hoshida et al.³² Cirrhosis, which is the most severe grade of fibrosis, is the main risk factor for HCC,¹⁸ and it is highly likely that most of the subjects with HCC in the study by Miki et al.¹⁴ had also cirrhosis. However, the investigators did not use subjects with HCV-related cirrhosis without HCC as control group, so it is likely that the association they found actually refers to severe fibrosis rather than to HCC.

Our results are different from those reported by a previous study in Asian subjects in which *DEPDC5* rs1012068 was found to be associated with lower fibrosis stage.³³ However, that study was conducted only on a small and selected cohort of subjects who underwent hepatectomy for HCC. Furthermore, there was no replication cohort, and the association analysis on fibrosis was performed differently. On the other hand, our data are in line with a recent study on HCV-positive subjects from Saudi Arabia in which homozygotes for *DEPDC5* rs1012068 were found to have a 1.7-fold higher risk of HCV-related cirrhosis.³⁴

The rs1012068 is an intronic variant of *DEPDC5*,¹⁴ and the role of *DEPDC5* in hepatic fibrogenesis is currently unknown. Therefore, to test association of the *DEPDC5* locus with the severity of liver fibrosis by an independent approach, we examined *DEPDC5* nonsynonymous variants. We specifically selected nonsynonymous variants because, leading to an amino acidic change, they are biologically more likely to result in a functional change of the protein. To increase the power of our study, we examined whether carriage of at least one nonsynonymous variant increased the risk of fibrosis in the pooled cross-sectional discovery and validation cohort. We found that the presence of nonsynonymous variants increased the risk of moderate/severe fibrosis, suggesting that *DEPDC5* might be involved in liver fibrogenesis. A limitation of this analysis is that two of the nonsynonymous variants are in linkage disequilibrium ($D' = 1$).

DEPDC5 rs1012068 does not affect gene expression.¹⁴ By using the data on linkage disequilibrium available from Ensembl genome browser, we found that *DEPDC5* rs1012068 is in linkage disequilibrium with two nonsynonymous variants (rs61731662 and rs146449468). We therefore speculate that the association found for the rs1012068 intronic variant might mirror the effect of one of these two amino acidic changes (and possibly others) on the protein structure and/or function or that this variant is associated with *DEPDC5* expression.

Other *DEPDC5* variants have been associated with familial focal epilepsy,³⁵⁻³⁷ but the function of the *DEPDC5* protein is still not known. *DEPDC5* has a DEP domain that is involved in different cellular functions, including Wnt signaling.^{26,27} Wnt/ β -catenin signaling is involved in liver fibrogenesis, inducing hepatic stellate cell activation.^{28,29} We showed that *DEPDC5* is highly expressed in immortalized LX-2 human hepatic stellate cells. We also showed that *DEPDC5* down-regulation increases β -catenin expression and induces MMP2 production in these cells. MMP2 is a downstream target of β -catenin.³⁸ In addition, several lines of evidence suggest that MMP2 is a profibrotic molecule: (1) it is highly expressed in liver fibrosis,³⁹ (2) it colocalizes with activated HSCs,⁴⁰ (3) it induces a remodeling of the extracellular matrix in favor of type 1 collagen,⁴¹ and (4) it may have an autocrine action inducing HSC activation.⁴²

We speculate that this noncoding variant, or the causal variant in linkage with it, determines a reduction in *DEPDC5* expression or activity. This reduction determines an increase in the Wnt/ β -catenin pathway

followed by an increase in the MMP2 synthesis, resulting in a higher degree of liver fibrosis.

In conclusion, *DEPDC5* variants increase fibrosis progression in European subjects with chronic HCV infection. Our findings suggest that *DEPDC5* down-regulation may contribute to HCV-related fibrosis by increasing MMP2 synthesis through the β -catenin pathway.

References

- Lavanchy D. Evolving epidemiology of hepatitis C virus. *Clin Microbiol Infect* 2011;17:107-115.
- Fattovich G, Giustina G, Degos F, Tremolada F, Diodati G, Almasio P, et al. Morbidity and mortality in compensated cirrhosis type C: a retrospective follow-up study of 384 patients. *Gastroenterology* 1997;112:463-472.
- Tremolada F, Casarin C, Alberti A, Drago C, Tagger A, Ribero ML, et al. Long-term follow-up of non-A, non-B (type C) post-transfusion hepatitis. *J Hepatol* 1992;16:273-281.
- Yano M, Kumada H, Kage M, Ikeda K, Shimamatsu K, Inoue O, et al. The long-term pathological evolution of chronic hepatitis C. *HEPATOLOGY* 1996;23:1334-1340.
- Poynard T, Mathurin P, Lai CL, Guyader D, Poupon R, Tainturier MH, et al. A comparison of fibrosis progression in chronic liver diseases. *J Hepatol* 2003;38:257-265.
- Poynard T, Bedossa P, Opolon P. Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups. *Lancet* 1997;349:825-832.
- Burza MA, Molinaro A, Attilia ML, Rotondo C, Attilia F, Ceccanti M, et al. PNPLA3 I148M (rs738409) genetic variant and age at onset of at-risk alcohol consumption are independent risk factors for alcoholic cirrhosis. *Liver Int* 2014;34:514-520.
- Estrabaud E, Vidaud M, Marcellin P, Asselah T. Genomics and HCV infection: progression of fibrosis and treatment response. *J Hepatol* 2012;57:1110-1125.
- Hu S, Zhao L, Yang J, Hu M. The association between polymorphism of P53 codon 72 Arg/Pro and hepatocellular carcinoma susceptibility: evidence from a meta-analysis of 15 studies with 3704 cases. *Meta Gene* 2013;1:126-137.
- He J, Yu G, Li Z, Liang H. Influence of interleukin-28B polymorphism on progression to hepatitis virus-induced hepatocellular carcinoma. *Tumour Biol* 2014;35:8757-8763.
- Guo PF, Jin J, Sun X. Influence of IL10 gene polymorphisms on the severity of liver fibrosis and susceptibility to liver cirrhosis in HBV/HCV-infected patients. *Infect Genet Evol* 2015;30:89-95.
- Sato M, Kondo M, Tateishi R, Fujiwara N, Kato N, Yoshida H, et al. Impact of IL28B genetic variation on HCV-induced liver fibrosis, inflammation, and steatosis: a meta-analysis. *PLoS One* 2014;9:e91822.
- Trépo E, Nahon P, Bontempi G, Valenti L, Falletti E, Nischalke HD, et al. Association between the PNPLA3 (rs738409 C>G) variant and hepatocellular carcinoma: evidence from a meta-analysis of individual participant data. *HEPATOLOGY* 2014;59:2170-2177.
- Miki D, Ochi H, Hayes CN, Abe H, Yoshima T, Aikata H, et al. Variation in the *DEPDC5* locus is associated with progression to hepatocellular carcinoma in chronic hepatitis C virus carriers. *Nat Genet* 2011;43:797-800.
- Kumar V, Kato N, Urabe Y, Takahashi A, Muroyama R, Hosono N, et al. Genome-wide association study identifies a susceptibility locus for HCV-induced hepatocellular carcinoma. *Nat Genet* 2011;43:455-458.
- Romeo S, Kozlitina J, Xing C, Pertsemlidis A, Cox D, Pennacchio LA, et al. Genetic variation in *PNPLA3* confers susceptibility to nonalcoholic fatty liver disease. *Nat Genet* 2008;40:1461-1465.
- Frazer KA, Murray SS, Schork NJ, Topol EJ. Human genetic variation and its contribution to complex traits. *Nat Rev Genet* 2009;10:241-251.
- Sangiovanni A, Prati GM, Fasani P, Ronchi G, Romeo R, Manini M, et al. The natural history of compensated cirrhosis due to hepatitis C virus: a 17-year cohort study of 214 patients. *HEPATOLOGY* 2006;43:1303-1310.
- Valenti L, Rumi M, Galmozzi E, Aghemo A, Del Menico B, De Nicola S, et al. Patatin-like phospholipase domain-containing 3 I148M polymorphism, steatosis, and liver damage in chronic hepatitis C. *HEPATOLOGY* 2011;53:791-799.
- Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. *HEPATOLOGY* 1996;24:289-293.
- Fischer J, Böhm S, Scholz M, Müller T, Witt H, George J, et al. Combined effects of different interleukin-28B gene variants on the outcome of dual combination therapy in chronic hepatitis C virus type 1 infection. *HEPATOLOGY* 2012;55:1700-1710.
- De Nicola S, Dongiovanni P, Aghemo A, Cheroni C, D'Ambrosio R, Pedrazzini M, et al. Interaction between PNPLA3 I148M variant and age at infection in determining fibrosis progression in chronic hepatitis C. *PLoS One* 2014;9:e106022.
- Marabita F, Aghemo A, De Nicola S, Rumi MG, Cheroni C, Scavelli R, et al. Genetic variation in the interleukin-28B gene is not associated with fibrosis progression in patients with chronic hepatitis C and known date of infection. *HEPATOLOGY* 2011;54:1127-1134.
- Ishak K, Baptista A, Bianchi L, Callea F, De Groote J, Gudat F, et al. Histological grading and staging of chronic hepatitis. *J Hepatol* 1995;22:696-699.
- Flicek P, Amode MR, Barrell D, Beal K, Billis K, Brent S, et al. Ensembl 2014. *Nucleic Acids Res* 2014;42:D749-D755.
- Wei W, Li M, Wang J, Nie F, Li L. The E3 ubiquitin ligase ITCH negatively regulates canonical Wnt signaling by targeting dishevelled protein. *Mol Cell Biol* 2012;32:3903-3912.
- Tauriello DV, Jordens I, Kirchner K, Slootstra JW, Kruitwagen T, Bouwman BA, et al. Wnt/ β -catenin signaling requires interaction of the Dishevelled DEP domain and C terminus with a discontinuous motif in Frizzled. *Proc Natl Acad Sci U S A* 2012;109:E812-E820.
- Li W, Zhu C, Li Y, Wu Q, Gao R. Mest attenuates CCl4-induced liver fibrosis in rats by inhibiting the Wnt/ β -catenin signaling pathway. *Gut Liver* 2014;8:282-291.
- Cheng JH, She H, Han YP, Wang J, Xiong S, Asahina K, et al. Wnt antagonism inhibits hepatic stellate cell activation and liver fibrosis. *Am J Physiol Gastrointest Liver Physiol* 2008;294:G39-G49.
- Xu L, Hui AY, Albanis E, Arthur MJ, O'Byrne SM, Blaner WS, et al. Human hepatic stellate cell lines, LX-1 and LX-2: new tools for analysis of hepatic fibrosis. *Gut* 2005;54:142-151.
- Friedman SL. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. *Physiol Rev* 2008;88:125-172.
- Hoshida Y, Fuchs BC, Tanabe KK. Genomic risk of hepatitis C-related hepatocellular carcinoma. *J Hepatol* 2012;56:729-730.
- Motomura T, Ono Y, Shirabe K, Fukuhara T, Konishi H, Mano Y, et al. Neither MICA nor *DEPDC5* genetic polymorphisms correlate with hepatocellular carcinoma recurrence following hepatectomy. *HPB Surg* 2012;2012:185496.
- Al-Anazi MR, Matou-Nasri S, Abdo AA, Sanai FM, Khan MQ, Albenmoussa A, et al. Variations in *DEPDC5* gene and its association with chronic hepatitis C virus infection in Saudi Arabia. *BMC Infect Dis* 2014;14:3839.
- Berkovic SF, Serratosa JM, Phillips HA, Xiong L, Andermann E, Díaz-Otero F, et al. Familial partial epilepsy with variable foci: clinical features and linkage to chromosome 22q12. *Epilepsia* 2004;45:1054-1060.
- Callenbach PM, van den Maagdenberg AM, Hottenga JJ, van den Boogerd EH, de Coe RF, Lindhout D, et al. Familial partial epilepsy with variable foci in a Dutch family: clinical characteristics and confirmation of linkage to chromosome 22q. *Epilepsia* 2003;44:1298-1305.

37. Dibbens LM, de Vries B, Donatello S, Heron SE, Hodgson BL, Chintawar S, et al. Mutations in DEPDC5 cause familial focal epilepsy with variable foci. *Nat Genet* 2013;45:546-551.
38. Wu B, Crompton SP, Hughes CC. Wnt signaling induces matrix metalloproteinase expression and regulates T cell transmigration. *Immunity* 2007;26:227-239.
39. Preaux AM, Mallat A, Nhieu JT, D'Ortho MP, Hembry RM, Mavier P. Matrix metalloproteinase-2 activation in human hepatic fibrosis regulation by cell-matrix interactions. *HEPATOLOGY* 1999;30:944-950.
40. Milani S, Herbst H, Schuppan D, Grappone C, Pellegrini G, Pinzani M, et al. Differential expression of matrix-metalloproteinase-1 and -2 genes in normal and fibrotic human liver. *Am J Pathol* 1994;144:528-537.
41. Benyon RC, Arthur MJ. Extracellular matrix degradation and the role of hepatic stellate cells. *Semin Liver Dis* 2001;21:373-384.
42. Benyon RC, Hovell CJ, Da Gaca M, Jones EH, Iredale JP, Arthur MJ. Progelatinase A is produced and activated by rat hepatic stellate cells and promotes their proliferation. *HEPATOLOGY* 1999;30:977-986.

Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.28322/supinfo.