Arch Virol (2014) 159:3345–3351
DOI 10.1007/s00705-014-2209-x

ORIGINAL ARTICLE

Core amino acid variation at position 110 is associated with sustained virological response in Caucasian patients with chronic hepatitis C virus 1b infection

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Received: 22 May 2014 / Accepted: 19 August 2014 / Published online: 27 August 2014
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Abstract The aim of this study was to analyze the impact of core variations on sustained virological response (SVR) to pegylated interferon plus ribavirin (PEG-IFN/RBV) and its association with predictive factors of response in Caucasian patients infected with genotype 1 hepatitis C virus (HCV-1). Full-length core sequences were analyzed in 100 Caucasian HCV-1-infected patients who received therapy with PEG-IFN/RBV. The associations between variations in the core protein and SVR, as well as with predictors of SVR, were analyzed. Variations at core 62, 70 and 110 were selected as candidates. There were almost no variations at these positions among patients harboring HCV-1a. However, they were identified in 10 (30.3 %), 21 (63.6 %) and 13 (39.4 %) subjects with HCV-1b, respectively. Among the HCV-1b patients, 39.1 % individuals carrying core R62 and 70 % subjects with core R62G showed SVR ($p = 0.141$), and 66.7 % of HCV-1b patients harboring core R70 and 38.1 % with core R70Q achieved SVR ($p = 0.157$), whereas the rate of SVR was 70 % for individuals with core T110 and 15.4 % for those with core T110N ($p = 0.004$). No statistical interaction between core variations and IL28B genotype was observed. Patients with R70 showed higher median (interquartile range) baseline plasma levels of low-density-lipoprotein cholesterol (LDL-C) than those with R70Q (96 [86-118] mg/dL vs. 76 [54-88] mg/dL, $p = 0.014$). We concluded that a substitution at core 110 is associated with a lower rate of SVR in Caucasian HCV-1b-infected patients receiving PEG-IFN/RBV. Furthermore, the variation at the core 70 position is related to plasma levels of LDL-C in these patients.

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Introduction

Several studies have shown that host and viral genomic factors play a critical role in the response to treatment of hepatitis C virus (HCV) infection [1–5]. Among the most important host genomic factors are variations in the interleukin 28B (*IL28B*) gene, which have been identified as strong predictors of sustained virological response (SVR) to pegylated interferon plus ribavirin (PEG-IFN/RBV) in HCV-monoinfected patients and human immunodeficiency virus (HIV)/HCV-coinfected individuals [3–5]. Moreover, it has been reported that these variations will likely remain relevant in predicting SVR in patients receiving protease-inhibitor-based combinations against HCV infection [1, 6].

Regarding viral factors, previous studies have reported that substitutions in the viral NS5A and core proteins are also related to SVR [7–12]. Interestingly, the core protein, which is the most conserved viral protein, interacts with many cellular proteins [13]. These interactions have multiple effects on the infected cells, including the impairment of innate immunity through several mechanisms [13, 14]. This suggests that variations in the core protein could have important consequences. In the core gene, variations at positions 70 and 91 in HCV genotype 1b (HCV-1b) carriers are predictors of poor virological response, both to PEG-

IFN/RBV and to triple therapy including telaprevir [1, 7, 8]. However, most of these studies have been carried out in Asiatic HCV-infected patients bearing HCV-1b [7, 8, 12], and geographical and ethnic characteristics of patients seem to influence the selection of different viral substitutions in the core protein related to SVR. For example, a South American study found that variations at other positions (core 75, core 147 and core 158), but not core 70 or 91, were associated with SVR [15]. In addition, little is known about the association of variations in the core protein with other predictors of SVR, such as *IL28B* genotype, HCV viral load or the level of low-density lipoprotein cholesterol (LDL-C). For all these reasons, studies including other ethnic groups, HCV subtype 1a (HCV-1a) carriers, and a full-length analysis of the core sequence are needed in order to clarify these issues.

The aim of this work was to analyze the impact of core variations on SVR to PEGIFN/RBV, as well as the association of such substitutions with other known predictive factors of response, in HCV-infected Caucasian patients with genotype 1.

Patients and methods

Study population

From August 2000 to December 2010, 109 patients infected with HCV genotype 1 (HCV-1) who received their first therapy against HCV infection with PEG-IFN/RBV were prospectively followed at the Infectious Diseases Unit of a tertiary care center in Spain. A basal serum sample and whole-blood samples were collected from each patient and stored at −80 °C for subsequent determinations.

HCV therapy

All patients received PEG-IFN alfa-2a at a dose of 180 μg once per week or PEG-IFN alfa-2b at a dose of 1.5 μg/kg once per week, both in combination with RBV at a daily dose of 800-1200 mg during 48 weeks. At weeks 12 and 24, HCV therapy was prematurely discontinued in non-responders, defined as those individuals who did not reach at least 2 log10 reduction in HCV-RNA levels at week 12 of treatment or undetectable plasma HCV RNA at week 24. SVR was defined as undetectable serum HCV RNA 24 weeks after the completion of HCV therapy.

HCV viral load, HCV subtyping, and IL28B rs12979860 genotyping

The plasma HCV RNA load was measured using quantitative real-time PCR assays (Cobas TaqMan; Roche Diagnostic Systems Inc., Pleasanton, CA, USA) in basal serum samples. The HCV genotype was determined using a RT-PCR hybridization assay (Versant HCV Genotype 2.0 LIPA; Siemens, Tarrytown, NY, USA). The HCV subtype was later confirmed by sequencing of the full-length core gene (see below). For SNP genotyping, DNA was extracted using the MagNA Pure system (Roche Diagnostics Corporation, Indianapolis, IN 46 250, USA). SNP rs12979860 (*IL28B*) genotyping was done as reported previously [4]. The researchers responsible for genotyping were blinded to the remaining data from the patients.

Viral RNA isolation, cDNA synthesis and sequencing

RNA was extracted from basal serum samples, frozen at −80 °C, using the MagNA Pure system (Roche Diagnostics, Mannheim, Germany). The RNA that was obtained was denatured at 80 °C for 5 min and primed with random hexamers (Eurogentec). The reverse transcription reaction was performed for 90 minutes at 37 °C, using Mu-MLV reverse transcriptase (Eurogentec).

The core gene was amplified by nested PCR with the outer primers ES (5′ CGA AAG GCC TTG TGG TAC TG 3′, sense) and EA (5′ ATG CTT GAG TTG GAG CAG TCG 3′, antisense) and the internal primers IS (5′ TTG TGG TAC TGC CTG ATA GGG T 3′, sense) and IA (5′ CTC CCG AAC GCA GGG CAC 3′, antisense) to generate a 766-nucleotide amplicon. The first PCR was performed in a final volume of 25 μL containing 5 μL of cDNA, 0.4 μM each primer, 0.4 mM each dNTP, 2.5 μL of 10x KAPA Taq Buffer A, and 0.5 U of KapaTaq DNA polymerase (Kapa Biosystems, Boston, USA). PCR conditions were as follows: denaturation at 95 °C for 5 min, followed by 40 cycles at 95 °C for 35 s, 59 °C for 45 s and 72 °C for 60 s, and a final extension at 72 °C for 7 min. The second PCR was performed like the first one, but containing 4 μL of the first PCR product as the target. The second PCR conditions were as follows: denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 35 s, 53 °C for 45 s, and 72 °C for 60 s, and a final extension step of 72 °C for 7 min.

Nested PCR products were bi-directionally sequenced using standard capillary electrophoresis techniques.

The sequences obtained were compared with the prototype sequence of subtype 1a or 1b, i.e., HCV-1a H77 and HCV-1b- J [16, 17], respectively. A mutational analysis of the full-length core gene sequences was performed in order to detect positions related to treatment outcome. After visual inspection, the VESPA program [18] was used to search for signature patterns in the amino acid sequences of viruses infecting responders.

Statistical analysis

Frequencies were compared using the chi-square test or Fisher's test. The Student's t-test and the Mann-Whitney U test were used for comparing continuous variables. The Breslow-Day test was employed to test homogeneity of the odds ratios (ORs) in the stratification studies.

For the purpose of this analysis, SVR was assessed in an on-treatment approach, i.e., excluding those who voluntarily dropped out or discontinued therapy due to adverse events. Statistical analysis was carried out using the SPSS statistical software package release 19.0 (IBM SPSS Inc, Chicago, IL, USA).

Nucleotide sequence accession numbers

The GenBank accession numbers for the sequences obtained in this study are KF060663-KF060714 and KJ739737-KJ739786.

Ethical aspects

Written informed consent to participate in this study was obtained from all patients, and the study was designed and performed according to the Declaration of Helsinki and was approved by the Ethics Committee of Valme Hospital (Seville, Spain).

Results

Characteristics of the study population

Among the 109 patients included, the HCV core genes from a total of 100 (91.7 %) subjects were successfully sequenced. This sequenced population was our study population. All of them were Caucasian. Among those whose viruses were sequenced, 46 (46 %) were HIV/HCV-coinfected patients. The *IL28B* genotype distribution was TT in 15 (15 %), CT in 47 (47 %) and CC in 38 (38 %) subjects. After sequencing, 67 (67 %) patients turned out to bear HCV-1a, and 33 (33 %) HCV-1b. Forty-two (42 %) of these patients achieved SVR. Characteristics of the study cohort are shown in Table 1.

Core variations in HCV-1 subtypes

Visual inspection and VESPA mutational analysis in the viral samples identified three main variations that were possibly associated with SVR in the core region in patients infected with HCV-1b, but none were found in patients infected with HCV-1a. Those variations were R62G, R70Q and T110N/S, and all of them were observed, almost exclusively, in subjects infected with HCV-1b (Table 2).

Regarding other previously reported variations, substitution at core 60, 75, 91, 147 and 158 were present in only 0 (0 %), 5 (7.5 %), 2 (2.9 %), 2 (2.9 %) and 0 (0 %) patients infected with HCV-1a and 0 (0 %), 5 (15.1 %), 3 (9.1 %), 3 (9.1 %) 0 (0 %) and 2 subjects (6.1 %) infected with HCV-1b.

Relationship between core variations and SVR

Due to the frequency of variations at the different positions in each HCV-1 subtype (Table 2), the effect of core R62G, R70Q and T110N variations on SVR could only be assessed in patients with HCV-1b (Fig. 1). Nine (39 %) patients carrying core R62 and 7 (70 %) subjects with core R62G attained SVR (p = 0.141, OR = 3.630, 95 % CI = 0.740-17,812). Eight (66.7 %) of those individuals harboring core R70 and 8 (38.1 %) patients with core R70Q showed SVR (p = 0.157, OR = 3.250, 95 %

Table 1 Characteristics of the study population

Variable	All patients n = 100	No SVR n = 58	SVR n = 42	p
Age, years	41.3 (38.5-45.8)	41.2 (38.7-46.1)	42.1 (37.7-45.8)	0.571
Male gender, no. (%)	80 (80.0)	48 (82.8)	32 (76.2)	0.418
HIV infection, no. (%)	46 (46.0)	32 (55.2)	14 (33.3)	0.031
Advanced fibrosis, no. (%)	39 (41.9)	29 (54.7)	10 (25)	0.004
HCV genotype 1b, no. (%)	33 (33.0)	17 (29.3)	16 (38.1)	0.356
HCV viral load >600000 IU/mL, no (%)	75 (75.0)	47 (81.0)	28 (66.7)	0.10
T-Cho, mg/dL	176 (145-207)	165 (141-208)	179 (156-203)	0.306
HDL-Cho, mg/dL	50.5 (38.0-62.0)	48.0 (35.5-62.5)	52.0 (40.5-61.0)	0.692
LDL-Cho, mg/dL	90 (70-112)	83 (65-108)	97 (83-120)	0.046
TGL, mg/dL	111 (84-164)	112 (93-157)	108 (76-167)	0.387
rs12979860 CC, no. (%)	38 (38.0)	16 (27.6)	22 (52.4)	0.012*

Values of quantitative variables are median (quartile 2-quartile 3)

SVR, sustained viral response; T-Cho, total cholesterol; HDL-Cho, HDL cholesterol; LDL-Chol, LDL cholesterol; TGL, triglycerides

* Pearson chi-square (CC vs CT + TT)

Table 2 Frequency of core substitutions at positions 62, 70 and 110 according to HCV subtype (n = 100)

Core position	Subtype 1a (N = 67)	Subtype 1b (N = 33)	P
62 no. (%)			
R*	67 (100)	23 (69.7)	<0.001
G	0 (0)	10 (30.3)	
70 no. (%)			
R*	66 (98.5)	12 (36.4)	<0.001
Q	1 (1.5)	21 (63.6)	
110 no. (%)			
T*	63 (94)	20 (60.6)	<0.001
N	2 (3)	13 (39.4)	
S	2 (3)	0 (0)	

R, arginine; G, glycine; Q, glutamine; T, threonine; N, asparagine; S, serine

* Representing the wild-type amino acid for genotype 1

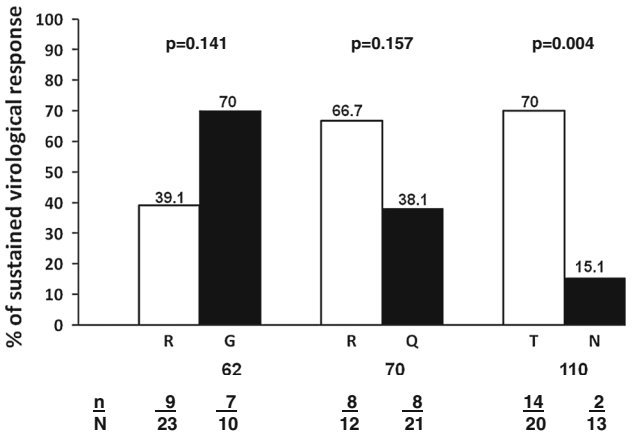

Fig. 1 Rate of sustained virological response according to the variations at core 62, 70 and 110 in patients infected with HCV-1b (n = 33). R, arginine; G, glycine; Q, glutamine; T, threonine; N, asparagine

CI = 0.733-14.402). Finally, SVR was observed in 14 (70 %) patients with core T110 and in 2 (15.1 %) subjects carrying core T110N (p = 0.004, OR = 0.078, 95 % CI = 0.013-0.464).

When HCV-1b-infected patients were separated according to the combination of the three positions described above for core, there were 5 (15.1 %) patients with the favorable combination (R62G, R70 and T110 [GRT]), and all of them reached SVR (p = 0.018, OR = 0.393, 95 % CI = 0.243-0.623).

Among patients harboring the *IL28B* genotype CC, 5 (83.3 %) patients with core R62G, 5 (71.4 %) individuals with R70 and 8 (88.9 %) subjects with T110 showed SVR. In the subgroup of individuals with an unfavorable *IL28B* genotype, 2 (50.0 %), 3 (60 %) and 6 (54.5 %) patients for R62G, R70 and T110, respectively, attained SVR. Is spite of this, no significant differences were observed in the effect sizes of each core variation between the *IL28B* CC and non-CC strata (Breslow-Day test p-values of 0.982, 0.254 and 0.845 for R62G, R70Q and T110 N variations, respectively).

Relationship between core variations and other predictors of SVR

In addition to HIV coinfection and *IL28B* genotype, other factors were associated with SVR in our population with a p-value <0.1, including LDL cholesterol, HCV viral load and advanced fibrosis (Table 1).

Patients with core R70 showed higher median (interquartile range) baseline plasma levels of LDL cholesterol (LDL-C) than those with core R70Q (96 [86-118] mg/dL vs. 76 [54-88] mg/dL, p = 0.014). However, no association was found between core 62 and core 110 substitutions and the plasma LDL-C level at baseline (data not shown). On the other hand, none of those core variations were associated with baseline HCV RNA load or with the presence of advanced fibrosis in the patients (data not shown).

Discussion

The results of this study suggest that the T110 N core variation is associated with SVR in Caucasian patients with HCV-1b, a finding that had not been reported so far. In addition, R70Q and R62G core variations in this study tended to be associated with SVR in the same group of patients. Moreover, the amino acid R70 is associated with higher plasma levels of LDL-C, a well-defined predictor of SVR to PEG-IFN/RBV treatment.

The results presented in this study raise a paradox, since core R70Q and core T110N, which lead to lower SVR rates, are much more frequent in HCV-1b patients, which, in general, respond better than HCV-1a patients to HCV therapy with PEG-IFN/RBV [19]. Therefore, according to our results, the poorer rates of SVR observed in HCV-1a carriers cannot be exclusively attributed to variations in the core gene. Likewise, this finding suggests that many positions in the HCV genome may be involved in the SVR to HCV therapy, as it had been observed previously in the IFN-sensitivity-determining region of NS5A [9–11].

We found that variations at core position 110 provide valuable information about the prediction of SVR to treatment with PEG-IFN/RBV in patients with HCV-1b. This finding could be also relevant for predicting SVR in the era of triple therapy against HCV infection [6, 20–22]. Thus, substitutions in the core protein may be useful to

identify patients who are very likely to respond to bitherapy with PEG-IFN/RBV and therefore may be treated with such a dual combination. This would reduce costs and, in the case of HIV/HCV coinfection, prevent drug interactions related to the use of directly acting antivirals (DAA) associated with PEG-IFN/RBV. In this way, determination of core mutations could be useful in clinical practice, since DAA-based therapy is not going to be massively available to all patients in all countries. In addition, the determination of core variations along with other predictors such as *IL28B* genotype could perhaps also help us to shorten the treatment duration in populations with a high likelihood of achieving SVR to bitherapy. Nevertheless, more studies in larger populations should be carried out to assess, in particular, how variations in the core protein affect SVR in patients receiving DAA, relating to *IL28B* polymorphisms and populations with different ethnicities.

Previously, core 110 had been related to SVR, but in individuals with HCV-2a [23]. This is a surprising result, since, to our knowledge, it has been not reported so far that the same core variation was associated with SVR in different HCV genotypes. On the other hand, our study suggests that variations in the HCV core gene vary according to ethnicity and geographic origin. Variation at core 110 could therefore be a marker of HCV-1b infecting our population. Similarly, other novel substitutions associated with HCV response have been observed in core protein from non-Japanese patients infected with HCV-1b [15, 24]. Therefore, our study should encourage the analysis of the complete core protein in order to detect new substitutions associated with SVR, especially in non-Asiatic patients.

In this study, variations at core 62 and 70 showed a tendency to be associated with SVR in HCV-1b-infected patients. Due to the low sample size, we cannot exclude a possible role of these two variations on the treatment response in our population. In previous studies, substitutions at core 70 have been related to several predictors of SVR and to liver damage [25–29], and in this study, an association was found between substitution at core 70 and baseline plasma levels of LDL-C. This result was in agreement with the results presented by Aizawa et al., who reported a relationship between variations at core 70 and LDL-C levels [30]. This suggests that these core substitutions are closely related to regulation of lipid metabolism in HCV-infected patients.

This study has the limitation that the number of sequences analyzed was relatively low. Because of this, it was not possible to apply multivariate analysis for testing if variation at position 110 is an independent factor associated with SVR. Therefore, it is possible that other predictors of SVR present in the study population could have influenced the analysis of core variations. Similarly, although we observed that all patients with the viral genotype combination R62G, R70 and T110 reached SVR, indicating a synergistic effect of these variations on achievement of SVR, it was not possible to apply general linear models to test evidence of interaction between these markers. However, since the effect size of the T110 variation is higher than that observed for the combination of the favorable core substitutions, the effect of this combination could be due to the inclusion of the T110 variation in the combination. Moreover, no statistical interaction was observed between core variations and the *IL28B* genotype in the stratified analyses, but this could be due to lack of statistical power. Therefore, another study with a larger number of patients will be necessary in order to confirm these results and to adjust the effect of HCV core variations for other variables more accurately.

In conclusion, a core substitution may predict the response to HCV therapy in HCV-1b-infected patients. It could be useful in clinical practice to identify patients in whom therapy with DAA could be spared and, perhaps, along with other predictors of SVR, subjects in whom HCV therapy can be shortened. Nevertheless, further studies including more patients are necessary to confirm our results.

Acknowledgments This work was supported by the Red de Investigación en SIDA (ISCIII-RETIC RD06/006 and RD12/0017), the Ministerio de Sanidad y Servicios Sociales (EC11-304-MJA and JG), and the Fundación para la Investigación y la Prevención del Sida en España (121004/10). KN is the recipient of a Sara Borrell postdoctoral perfection grant from the Instituto de Salud Carlos III (grant number SCO/523/2008). CC is the recipient of a grant from the Ministerio de Ciencia e Innovación of Spain (PTA 2001-5503-I). JM is the recipient of a grant from the Servicio Andaluz de Salud de la Junta de Andalucía (B-0037). JAP is the recipient of an intensification grant from the Instituto de Salud Carlos III (grant number Programa-I3SNS).

Conflict of interest The authors declare that they have no conflict of interest.

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