

## Observational Study

## Impact of hepatitis C virus core mutations on the response to interferon-based treatment in chronic hepatitis C

Camelia Sultana, Gabriela Opreșan, Monica Delia Teleman, Sorin Dinu; HepGen 88/2012 Project Team; Cristiana Oprea, Mihai Voiculescu, Simona Ruta

Camelia Sultana, Simona Ruta, Virology Discipline, Carol Davila University of Medicine and Pharmacy, 030304 Bucharest, Romania

Camelia Sultana, Simona Ruta, Emergent Diseases Department, Stefan S. Nicolau Institute of Virology, 030304 Bucharest, Romania

Gabriela Opreșan, Faculty of Pharmacy, Titu Maiorescu University, 030304 Bucharest, Romania

Gabriela Opreșan, Sorin Dinu, Molecular Epidemiology Laboratory, NIRDMI Cantacuzino, 030304 Bucharest, Romania

Monica Delia Teleman, Department of Microbiology and Epidemiology, Carol Davila University of Medicine and Pharmacy, 030304 Bucharest, Romania

Cristiana Oprea, Victor Babeș Clinic of Infectious and Tropical Diseases, 030304 Bucharest, Romania

Mihai Voiculescu, Fundeni Institute, 030304 Bucharest, Romania

**Author contributions:** Sultana C, Opreșan G, Ruta S contributed to study conception and design; Oprea C and Voiculescu M contributed to patients recruitment and treatment and data acquisition; HepGen 88/2012 Project Team contributed to data acquisition; Sultana C, Opreșan G, Teleman MD, Dinu S and Ruta S contributed to virological testing, data analysis and interpretation, and drafted the manuscript; Sultana C and Ruta S wrote, edited and reviewed the final form of the manuscript; all authors approved the final form of the article.

**Supported by** PN-II-PT-PCCA-2011-3.2 Program, No. 88/2012; HepGen “Investigation of viral and host markers of non-response to anti-viral treatment in chronic hepatitis C” funded by the Romanian Ministry of Education and Research.

**Institutional review board statement:** The study was reviewed and approved by the Institutional Review Board IRB comitee of the “Stefan S. Nicolau” Institute of Virology, Bucharest, Romania.

**Informed consent statement:** All study participants provided informed written consent prior to study enrollment.

**Conflict-of-interest statement:** There are no conflicts of interest to report.

**Data sharing statement:** No additional data are available.

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**Manuscript source:** Invited manuscript

**Correspondence to:** Simona Ruta, MD, PhD, Professor, Chair of Virology, Emergent Diseases Department, Stefan S. Nicolau Institute of Virology, 285 Mihai Bravu Bvd, 030304 Bucharest, Romania. [simona@simonaruta.ro](mailto:simona@simonaruta.ro)  
**Telephone:** +40-213242590  
**Fax:** +40-213242590

**Received:** June 24, 2016

**Peer-review started:** June 28, 2016

**First decision:** July 29, 2016

**Revised:** August 9, 2016

**Accepted:** August 23, 2016

**Article in press:** August 23, 2016

**Published online:** October 7, 2016

### Abstract

#### AIM

To determine whether hepatitis C virus (HCV) core substitutions play a role in the response to interferon-

based treatment in Caucasian patients.

## METHODS

One hundred eight HCV chronically infected patients initiating treatment with pegylated IFN plus ribavirin for 48 wk were tested for baseline substitutions at codons 70 and 91 of the viral core protein (BigDye Terminator vers.3.1, Applied Biosystems,) and for genetic polymorphisms in host *IL28B* gene rs12979860 (Custom TaqMan 5' allelic discrimination assay; Applied Biosystems).

## RESULTS

Of the patients, all were infected with HCV genotype 1b, 44.4% had low baseline HCV viral load, and 37.9% had mild/moderate fibrosis. Only 38.9% achieved therapeutic success, defined as sustained virological response (SVR). Eighty-eight percent of the patients presented at least one substitution at core position 70 (R70Q/H) or/and position 91 (L91M). The favorable *IL28B* CC polymorphism was detected in only 17.6% of the patients. In the univariate analysis, young age ( $P < 0.001$ ), urban residence ( $P = 0.004$ ), *IL28B* CC genotype ( $P < 0.001$ ), absence of core mutations ( $P = 0.005$ ), achievement of rapid virologic response ( $P < 0.001$ ) and early virological response ( $P < 0.001$ ) were significantly correlated with SVR. A multivariate analysis revealed three independent predictors of therapeutic success: young age ( $P < 0.001$ ), absence of core substitutions ( $P = 0.04$ ) and *IL28B* CC genotype ( $P < 0.001$ ); the model correctly classified 75.9% of SVR cases with a positive predictive value of 80.7%.

## CONCLUSION

HCV core mutations can help distinguish between patients who can still benefit from the affordable IFN-based therapy from those who must be treated with DAAs to prevent the evolution towards end-stage liver disease.

**Key words:** Chronic hepatitis C; Caucasian patients; Core substitutions; *IL28B* polymorphism; Treatment

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**Core tip:** The high cost of the newly introduced direct acting antivirals precludes universal replacement of the suboptimal interferon-based therapy for chronic hepatitis C. Therefore, a series of host- and virus-related factors are used as prognostic markers of treatment response. In Asian patients, a newly described viral factor is represented by amino acid substitutions in the hepatitis C virus core protein at positions 70 and 91. The present study confirms that core substitutions are also found in Caucasian patients and, together with age and *IL28B* genotype, can be used as predictors of the outcome of interferon-based therapy.

Sultana C, Opreșan G, Teleman MD, Dinu S; HepGen 88/2012 Project Team; Oprea C, Voiculescu M, Ruta S. Impact of hepatitis C virus core mutations on the response to interferon-based treatment in chronic hepatitis C. *World J Gastroenterol* 2016; 22(37): 8406-8413 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i37/8406.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i37.8406>

## INTRODUCTION

Hepatitis C might become the first curable chronic disease due the remarkable efficacy of the newly introduced direct acting antiviral drugs (DAAs). Interferon-free regimens, based on combinations of DAAs with pan-genotypic activity, allow for shorter courses of treatment without severe side effects<sup>[1]</sup>. Nevertheless, the high cost of DAAs continues to preclude universal replacement of the classic treatment consisting of PEGylated-interferon and ribavirin (PEGIFN/RBV). This therapeutic combination is effective in approximately 50% of hepatitis C virus (HCV) chronically infected patients, with the response rate strongly dependent on the infecting genotype<sup>[2]</sup> and correlated with a series of other viral and host factors, *e.g.*, baseline/on-treatment viral load, liver fibrosis, host *IL28B* polymorphisms<sup>[3-6]</sup>.

Across the 9.6 kb genome of HCV, several regions (specifically HVR1, IFN sensitivity-determining region and an IFN/ribavirin resistance-determining region in NS5A<sup>[7,8]</sup>) have been extensively analyzed in relation to treatment outcome, whereas the more conserved core gene has been mostly used for HCV genotyping and classification. Nevertheless, the core region has been reported to antagonize the antiviral response induced by IFN by interacting with the IFN-activating and signaling pathways<sup>[9,10]</sup>. Substitutions in certain less conserved sites of the core region can give rise to viral quasispecies resistant to interferon treatment<sup>[11]</sup>. Several reports, mainly from Japan, have indicated that amino acid substitutions in positions 70 and 91 of the core protein are associated with the outcome of interferon-based therapy<sup>[12,13]</sup>.

Because most of the studies related to these core mutations have been conducted in Asian populations, the aim of the present study is to determine whether HCV core substitutions are present and play a significant role in the outcome of interferon-based treatment in Caucasian patients, as well to inform better selection and prioritization of those patients who can still benefit from this affordable therapy.

## MATERIALS AND METHODS

### Study population

An observational study was conducted on 108 HCV chronically infected Caucasian patients treated for the

**Table 1** Demographic, clinical and virological characteristics of study patients *n* (%)

Characteristics	Total <i>n</i> = 108	IL28B CC <i>n</i> = 19	IL28B non-CC <i>n</i> = 89	<i>P</i> value
Age (yr), median	53.5 (22.0-68.3)	48.2 (22.1-66.4)	53.8 (22.1-68.3)	0.137
Female	64 (59.3)	8 (42.1)	56 (62.9)	0.094
Residence urban	78 (72.2)	17 (87.5)	61 (68.5)	0.090
Baseline ALT (mg/dL), median	76.5 (16-890)	103 (16-890)	74 (17-256)	0.031
Baseline HCV-RNA (log <sub>10</sub> IU/mL), median	6.1 (4.15-7.3)	6.2 (4.15-7.2)	6.1 (4.15-7.3)	0.333
Mild/moderate fibrosis	38 (58.5)	9 (69.23)	29 (55.8)	0.470
Absence of core mutations - DW	13 (12.0)	6 (31.6)	7 (7.9)	0.011
RVR (%)	14 (13.0)	7 (36.8)	7 (7.9)	0.003
EVR (%)	43 (39.8)	16 (84.2)	27 (30.3)	< 0.001
SVR (%)	42 (38.9)	17 (89.5)	25 (28.1)	< 0.001

HCV: Hepatitis C virus; DW: Double wild-type; SVR: Sustained virological response; EVR: Early virological response; RVR: Rapid virological response.

first time with a combination of PEGylated IFN- $\alpha$ 2a (180  $\mu$ g per week) or PEGylated IFN- $\alpha$ 2b (1.5  $\mu$ g/kg per week) plus ribavirin (1000 or 1200 mg, dependent on body weight) in two tertiary care facilities in Bucharest, Romania. All patients met the following inclusion criteria: 18-65 years of age, detectable HCV viremia, and previously untreated. The exclusion criteria were: HBV or HIV co-infections' malignancies; coexistent liver disease of other etiology; organ recipients, clinically significant pulmonary, renal, cardiovascular or hematological diseases; current pregnancy and lactation. Each patient provided informed consent and the Bioethics Committee of the Institute of Virology approved the study.

#### Measurement of HCV-RNA

HCV-RNA was performed at baseline, weeks 4 and 12 of treatment and 24 wk after treatment completion using COBAS AmpliPrep/COBAS TaqMan Quantitative Test, version 2.0 (Roche Diagnostics GmbH, Germany) with a linear range of HCV-RNA quantification between 15 and 100000000 IU/mL. Rapid virologic response (RVR) was defined as undetectable HCV-RNA at week 4 of treatment. Early virological response (EVR) was defined as undetectable HCV-RNA at week 12 of treatment, and sustained virological response (SVR) was defined as undetectable HCV-RNA 6 mo after treatment completion.

#### Viral genotyping and detection of substitutions at codons 70 and 91 in core protein

Viral RNA was extracted from 140  $\mu$ L serum using a commercial kit (QIAamp Mini Viral Kit, Qiagen). Reverse transcription was performed as described previously<sup>[14]</sup> and the cDNA was used in a semi-nested PCR yielding a 422 bp amplicon spanning the HCV core region<sup>[14,15]</sup>. The amplicons were sequenced (BigDye Terminator v3.1 and 3130 Genetic Analyzer, Applied Biosystems) and the resulting sequences were edited with BioEdit version 7.0.5.3<sup>[16]</sup> and used for genotyping (NCBI BLAST) and to assess the presence of substitutions at positions 70 and 91 in the core protein.

#### Genetic polymorphism in the IL28B gene (rs12979860)

Genetic polymorphism in the *IL28B* gene (rs12979860) was investigated using Custom TaqMan 5' allelic discrimination assay (Assays-by-DesignSM Service for SNP Genotyping Assays, Applied Biosystems, United States) and running a real time PCR on an ABI 7300 instrument with primers and fluorescent probes predesigned by the manufacturer and interpreted using SDS software from Applied Biosystems Inc., United States.

#### Liver fibrosis

Liver fibrosis was assessed using a noninvasive method - transient elastography (FibroScan™) - that discriminated between mild/moderate fibrosis (F1 + F2) and advanced fibrosis (F3 + F4) by assigning a value of liver stiffness lower or higher than 9.5 kPa<sup>[17]</sup>.

#### Statistical analysis

Statistical analysis performed with IBM SPSS Statistics version 20. Univariate analysis was performed for both categorical and continuous variables; *P* values were calculated using the independent samples Mann-Whitney *U* test for continuous variables and Pearson  $\chi^2$  or Fisher's exact test for categorical variables. Variables with statistical significance (*P* < 0.05) in the univariate analysis were introduced into a multivariate logistic regression model.

## RESULTS

#### Characteristics of patients and response to treatment

Patients' characteristics are summarized in Table 1. The median baseline HCV-RNA was 6.1 log<sub>10</sub>IU/mL. Of the 108 patients, 44.4% had a baseline HCV viral load lower than 600000 IU/mL (5.8 log<sub>10</sub>IU/mL) and 37.9% had mild or moderate fibrosis. All patients were infected with HCV genotype 1b. Only 38.9% (42 patients) achieved SVR, and modest percentages had prompt responses during therapy: 13% had RVR and 39.8% had EVR.

The favorable *IL28B* CC genotype was detected in only 17.6% of the patients, and had no significant

**Table 2** Patients characteristics related to core mutation type, univariate analysis

Characteristics	Patients with DW-type infection <i>n</i> = 13	Patients with R70Q/H and/or L91M substitutions <i>n</i> = 95	Patients with L91M substitution <i>n</i> = 40	Patients with R70Q/H substitution <i>n</i> = 8	<i>P</i> value <sup>1</sup>
Age (yr) median	51.5 (29.1-66.54)	53.6 (22.1-68.3)	53.3 (24.4-67.3)	50 (33.8-58.8)	a = 0.607 b = 0.694 c = 0.860
Gender Female (%)	7 (53.8)	57 (60)	22 (55)	5 (62.5)	a = 0.767 b = 1.000 c = 1.000
Residence Urban (%)	11 (84.6)	67 (70.5)	33 (82.5)	5 (62.5)	a = 0.509 b = 1.000 c = 0.325
Baseline ALT (mg/dL) median	94 (3-235)	76 (16-890)	78 (17-890)	67 (54-197)	a = 0.592 b = 0.849 c = 1.000
Baseline HCV-RNA (log <sub>10</sub> IU/mL), median	6.25 (4.15-6.9)	6.05 (4.15-7.3)	6.05 (4.1-7.3)	6.3 (5.2-7.1)	a = 0.966 b = 1.000 c = 0.414
Mild/moderate fibrosis (F1 + F2, %)	4 (57.1)	34 (58.6)	14 (58.3)	4 (57.14)	a = 0.881 b = 0.925 c = 0.283
IL28B CC (%)	6 (46.2)	13 (13.7)	8 (20.0)	1 (12.5)	a = 0.011 b = 0.080 c = 0.174
RVR (%)	6 (46.2)	8 (8.4)	4 (10.0)	0 (0.0)	a = 0.002 b = 0.009 c = 0.005
EVR (%)	10 (76.9)	33 (34.7)	15 (37.5)	3 (37.5)	a = 0.005 b = 0.024 c = 0.164
SVR (%)	10 (76.9)	32 (33.7)	15 (37.5)	2 (25.0)	a = 0.005 b = 0.024 c = 0.032

<sup>1</sup>*P* value symbols a, b, c: cases from each category type of viral substitutions (columns 3, 4, 5) vs cases without viral mutation [double wild-type (DW)-type - column 2]. HCV: Hepatitis C virus; SVR: Sustained virological response; EVR: Early virological response; RVR: Rapid virologic response.

correlation with patients' demographic characteristics (age, gender, urban residence; Table 1).

Patients with *IL28B* genotype CC had the highest therapeutic success rates than those with TT or CT genotypes for the following outcomes: RVR (36.8% vs 7.9%, OR = 6.8, *P* = 0.003); EVR (84.2% vs 30.3%, OR = 12.2, *P* < 0.001), and SVR (89.5% vs 28.1%, OR = 21.8, *P* < 0.001).

#### Impact of core mutations on the treatment response

According to the sequencing results, only 12% of the patients were infected with double wild-type (DW) strains - defined as presence of arginine (R) and leucine (L) at core position 70 and 91, respectively - while the rest had glutamine/histidine at position 70 (R70Q/H), or/and methionine at position 91 (L91M). Of the patients, R70Q/H substitution was present in viral isolates infecting 7.4%, and L91M was observed in 37%, while patients displaying both mutations represented 43.5% of the study population.

The presence of any substitutions at positions 70 and 91 of the core protein was associated with lower rates of RVR, EVR, and SVR (Table 2). Patients infected with DW-type strains obtained SVR more frequently than patients with R70Q/H substitution (76.9% vs 37.5%, OR = 5.6, *P* = 0.032), or L91M substitution

(76.9% vs 25.0%; OR = 12, *P* = 0.032). Furthermore, looking simultaneously at both mutation sites, patients infected with HCV DW-type strains obtained SVR significantly more frequently than patients with substitution at any 70 or 91 positions (76.9% vs 33.7%, *P* = 0.032; OR = 6.6, *P* = 0.005).

No significant differences related to demographic characteristics or virological parameters (HCV viremia, ALT, liver fibrosis) were detected between the patients infected with DW-type strains and those with R70Q/H and L91M substitutions (Table 2).

#### Predictive factors for treatment success

In the univariate analysis, young age, urban residence, *IL28B* CC genotype, absence of core mutations and achievement of RVR and EVR were significantly correlated with the rate of therapeutic success, defined as SVR (Table 3).

Direct logistic regression was performed to assess the impact of different viral and host factors on the likelihood of achieving SVR, and the overall model contained all predictors with statistically significance in the univariate analysis. In the multivariate analysis, young age (less than 50 years old), absence of any type of core mutations, and presence of *IL28B* CC genotype were independently associated with

**Table 3 Predictive factors for sustained virological response, univariate analysis *n* (%)**

Variable	SVR <i>n</i> = 42	No SVR <i>n</i> = 66	<i>P</i> value
Age (yr), median	48.1 (22.1-68.3)	54.7 (22.1-67.3)	< 0.001
Female gender	22 (52.4)	42 (63.6)	0.246
Urban residence (%)	37 (88.1)	41 (62.1)	0.004
Baseline ALT (mg/dL), median	79 (16-890)	75 (17-231)	0.688
Baseline HCV-RNA, median	6.2 (4.15-7.3)	6.2 (4.8; 7.3)	0.167
Mild/moderate fibrosis	18 (75.0)	20 (48.8)	0.107
IL28B type CC (%)	17 (40.5)	2 (3.0)	< 0.001
Absence of core mutations - DW	10 (23.8)	3 (4.5)	0.005
Presence of any core mutation, (%)	32 (76.2)	63 (95.5)	0.005
Viral mutation in core 91 only (%)	15 (60.0)	25 (84.3)	0.024
Viral mutation in core 70 only (%)	2 (16.7)	6 (66.7)	0.032
RVR (%)	13 (31.0)	1 (1.5)	< 0.001
EVR (%)	37 (88.1)	6 (9.1)	< 0.001

HCV: Hepatitis C virus; DW: Double wild-type; SVR: Sustained virological response; EVR: Early virological response; RVR: Rapid virologic response.

**Table 4 Multivariate analysis on independent predictive factors for sustained virological response**

Variable	Adjusted OR	95%CI	<i>P</i> value
Age (< 50 yr)	0.976	0.967-0.986	< 0.001
Absence of core mutations	4.710	1.10-21.30	0.04
IL28B polymorphism	19.200	4.05-91.40	< 0.001

achieving SVR (Table 4).

This multivariate model correctly classified 75.9% of cases with a positive predictive value of 80.7%.

## DISCUSSION

We report that the absence of substitutions in core positions 70 and 91 is a good predictor for achieving SVR after PEG-IFN/RBV treatment in Caucasian patients; together with *IL28B* polymorphisms and age, this absence can be used to stratify HCV-infected patients according to the likelihood of response to a currently suboptimal, but affordable, interferon-based therapy.

The success rate of IFN-based therapy was rather low in the present cohort, despite the fact that several baseline predictors suggested a promising patient profile (relatively young age (median: 53.5 years), low baseline HCV viral load in half of the cases, and minimal fibrosis in more than one-third of the cases). Nevertheless, the on-treatment viral kinetic response was modest, with a minority of patients achieving undetectable viral replication after 4 wk of therapy. An analysis of *IL28B* polymorphism rs12979860, the most important predictor of SVR in patients without RVR<sup>[18]</sup>, revealed that non-CC *IL28B* genotypes were predominant (82.4%). These variants are associated with endogenous activation of the innate immune responses, higher baseline expression levels of IFN-stimulated genes, and a constant activation of the IFN signaling pathway that renders patients unresponsive to IFN treatment<sup>[18,19]</sup>. The exclusive presence of subtype 1b and the low prevalence of the favorable

CC genotype can explain, at least in part, the low rate of virological response, but a series of others factors must be taken into consideration: questionable or inconsistent patient adherence to treatment, presence of adverse events that could determine temporary treatment interruptions (none were acknowledged by the study patients), and preexisting mutations in other genomic regions that could render the virus less susceptible to the prescribed drugs. In this study, core protein substitutions at positions 70 and 91 were present in viral isolates infecting 88% of the patients and linked with a significantly decreased probability of achieving SVR.

There has been increased evidence that substitutions at position 70 in the core protein are found regularly in Asian HCV 1b infected patients, with the mutant clone R70Q detectable even in newly infected people and no distinguishing characteristics for the mutant strain in terms of viral fitness or demographic distribution<sup>[20]</sup>. Studies investigating HCV core mutations in Caucasian patients are scarce, but two studies of very limited numbers of patients have suggested an association between R70 and an increased response to therapy<sup>[21,22]</sup>.

In our study, we detected high rates of core protein substitutions at positions 70 and 91 in 108 Caucasian patients infected with HCV subtype 1b; these substitutions had no significant association with viral load, but were significantly associated with a low therapeutic success rate. Our results are in accordance with previous reports that indicated an absence of substitutions at positions 70 and 91 in core protein as a significant predictor for the success of IFN-based therapy<sup>[12,21,22]</sup>. These results have also been confirmed *in vitro*; Funaoka *et al.*<sup>[23]</sup> evaluated the effect of interferon-alpha on HCV core mutants (R70Q/H and L91M) in terms of viral replication and response to treatment and found a significantly higher degree of IFN resistance compared to the wild-type virus, associated with decreased expression of the IFN-stimulated genes. The proposed hypothesis for the interferon resistance of core mutants was related to the inhibition of the interferon signaling pathway,

potentially involving SOCS3 (suppressor of cytokine signaling). These proteins are stimulated by various cytokines including IL6, which was upregulated in cells transfected with a core mutant. This mechanism can be observed *in vivo* as well, as chronically HCV infected patients have increased levels of inflammatory cytokines, including IL-6 and TNF-alpha<sup>[24]</sup>.

Another interesting mechanism that might explain the role of core substitutions in interferon resistance is their potential influence on the expression of minicore proteins- isotypes of the normal core protein, that lack an N-terminal segment<sup>[25]</sup>. Two important minicore proteins terminate in the vicinity of amino acids 70 and 91; consequently, any structural changes in these amino acids can alter the expression of minicore proteins and implicitly the HCV functioning and IFN sensitivity<sup>[11]</sup>.

To our knowledge, this is one of the first studies conducted on Caucasian patients that extend and confirm the results obtained in Asian populations related to the impact of amino acid substitution in the HCV core region on treatment response. Although our study does not involve a very large number of patients, further sampling is unlikely, as the clinical facilities investigated in this study treat subjects from all over the country. Performing mathematical modeling of the cost-effectiveness of sequencing for HCV core mutations would be beneficial. Although the cost of this test may be rather high, it is significantly lower than the prohibitive cost of DAAs. Several recent studies have attempted to provide an estimate on the cost-effectiveness of interferon-free regimens (assuming a price of \$100000 and a success rate of 90%). Their results support a delay in treatment for patients with mild degrees of fibrosis<sup>[26-28]</sup>. As such, the potentially beneficial role of core sequencing in selecting patients from this subclass who are responsive to interferon-based therapy may outweigh the potential price limitation. This is particularly true for at this time, when there is a constant need for ethical, evidence-based criteria for the prioritization of interferon-free treatment in countries that cannot yet afford the universal introduction of the new highly active antivirals. In addition, there have been reports that HCV core substitutions can also predict the primary outcomes of therapy using first generation protease inhibitors<sup>[29]</sup> and that the *IL28B* genotype is furthermore predictive of the response to triple therapy in patients infected with HCV genotype 1<sup>[30]</sup>. Consequently, future studies will be needed to extend our results to patients treated with the novel categories of antivirals recently introduced for the treatment of HCV chronic infection.

Moreover, recent studies have also indicated that HCV core substitutions are involved in the progression of chronic hepatitis C to hepatocellular carcinoma (HCC)<sup>[31]</sup>. The R70Q variant has been associated with an increased malignancy risk<sup>[32-34]</sup>, while the implication of core 91 substitution was dubitable<sup>[11]</sup>. A

very recent study using deep-sequencing reported that the presence of baseline HCV strains harboring more than 42% non-R70 quasispecies or more than 98.5% non-L91 mutants was associated with an increased HCC risk<sup>[35]</sup>. As long as the residual risk for HCC development in compensated cirrhotic patients with SVR after IFN treatment remains quite high (3.4% at 5 years and 23.7% at 20 years<sup>[36]</sup>), the impact of core mutations on the transforming capacity of HCV core protein is worth studying.

In conclusion, this study reports absence of core genomic mutations associated with IL28B CC polymorphism as prognostic markers for a favorable outcome in HCV chronically infected Caucasian patients treated with interferon-based regimens. Core genomic mutations can be used to tailor treatment and distinguish between those patients who can respond to the affordable bi-therapy and those who must be urgently treated with DAAs to prevent evolution towards end-stage liver disease or HCC.

## ACKNOWLEDGMENTS

**HepGen 88/2012 Project Team:** Cantacuzino National Institute for Research and Development in Microbiology and Immunology: Maria Condei; Monica Straut; Codruta Usein; Mihaela Oprea; "Victor Babeş" Clinic of Infectious and Tropical Diseases, Bucharest; Prof. Petre Iacob Calistru; Alma Kosa; Gratiela Tardei; Claudia Leulescu; Angelica Nour; George Gherlan; Gh. Voiculescu; Simona Cazacu; Fundeni Clinical Institute: Mihai Voiculescu, Elena Rusu; Monica Ecobici; Laurentiu Micu; Diana Zilisteanu; Camelia Achim; Andreea Radasan; Mirela Miu; Emilia Grigore; Georgia Micu; Laura Panaiteanu; Paula Dragoescu; "St. Nicolau" Institute of Virology, Carmen Diaconu; Aura Temereanca; Claudia Dita; Petruta Mihaila; Personal Genetics: Georgeta Cardos; Bogdanka Militescu; Petruta Gurban; Antonie Edu; Gabriela Bucur; Cristina Ionescu; Pompilia Apostol; Vladimir Celmare; Sonia Spandole; Eugen Radu.

## COMMENTS

### Background

Interferon-free regimens, based on combinations of direct acting antivirals (DAAs) with pan-genotypic activity, have a remarkable efficacy and might transform hepatitis C into a curable chronic disease. Nevertheless, the high cost of DAAs still precludes the universal replacement of the classic PEGylated-interferon and ribavirin therapy (PEG-IFN/RBV) that is dependent on a series of host and viral factors. In Asian patients, a newly described viral factor influencing the outcome of bi-therapy treatment is represented by amino acid substitutions in hepatitis C virus (HCV) core protein positions 70 and 91.

### Research frontiers

There is very scarce information on the presence and significance of HCV core substitutions in Caucasian populations. Nevertheless, a number of studies have indicated that amino acid substitutions in the core protein play an important role in the very early dynamics of viral replication during bi-therapy and triple therapy of chronic hepatitis C as well as in the evolution toward hepatocellular carcinoma.

### Innovations and breakthroughs

This is one of the first studies confirming that HCV core substitutions are not specific to the Asian population, being also found in Caucasian patients. Moreover, it demonstrates that absence of core genomic mutations, together with young age and *IL28B* CC genotype, is a prognostic marker for favorable outcome in chronic HCV-infected Caucasian patients treated with PEG-IFN/RBV.

### Applications

The research hotspot is the identification of a new viral factor that can help distinguish between patients who can still benefit from the affordable IFN-based therapy from those who must be urgently treated with DAAs to prevent the evolution towards end-stage liver disease. This is an important practical instrument in countries with developing economies that cannot afford universal introduction of DAA because it can facilitate the prioritization of patients who will benefit from less expensive therapeutic regimens. Further application of these results can be derived from the recently reported role of core substitution in the progression of HCV infection to hepatocellular carcinoma. As long as a residual risk for HCC development persists even in patients successfully treated with interferon or with IFN-free regimens, the impact of core mutations on the transforming capacity of the HCV core protein is worth studying.

### Terminology

HCV core gene is a conserved part of the viral genome that is mostly used for HCV genotyping and classification. Nevertheless, the core region can antagonize the antiviral response induced by IFN, interacting with the IFN-activating and signaling pathways. Substitutions in less conserved sites (positions 70 and 91 in the core region) can contribute to resistance to interferon treatment.

### Peer-review

The manuscript is very well written and clearly states its aims and conclusions. It looks overall good with some limitations of low number of patients and low SVR. Questions were raised concerning the cost of core mutations testing. A paragraph responding to the study limitations and the necessity of core sequencing cost effectiveness evaluation was added by the authors in the revised version.

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ISSN 1007-9327



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