

RESPONSE FACTORS TO PEGYLATED INTERFERON-ALFA/RIBAVIRIN TREATMENT IN CHRONIC HEPATITIS C PATIENTS GENOTYPE 1B

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Abstract - Hepatitis C virus infection is the most common chronic blood-borne infection and one of the most important causes of chronic liver disease. Knowing the predictors associated with pegylated interferon/ribavirin (PEG-IFN/RBV) combination therapy response is important for evidence-based treatment recommendations. The goal of this study was to identify host and viral factors of response to PEG-IFN/RBV treatment in chronic hepatitis C genotype 1b patients. We have examined the relationship between gender, age, level of alanine aminotransferase (ALT), viral load and liver fibrosis progression on therapy response. ALT level and viral load were evaluated before starting treatment with combination therapy. The elevated levels of ALT and route of HCV transmission were found to be significantly associated with the response to therapy in HCV-infected patients. Our findings may be useful for estimating a patient's likelihood of achieving sustained viral response.

Key words: Hepatitis C virus (HCV), genotype 1b, (pegylated) interferon-alfa/ribavirin and response to therapy

INTRODUCTION

Chronic hepatitis C virus (HCV) infection is a global health problem affecting more than 180 million people worldwide, or approximately 3% of the human population (WHO, 1999). Chronic HCV infection frequently results in liver cirrhosis and is associated with an elevated risk of developing hepatocellular carcinoma (HCC) (Giannini et al., 2003). Until recently, pegylated interferon/ribavirin (PEG-IFN/RBV) has been the standard of care treatment for all hepatitis C genotypes, resulting in viral eradication in approximately 50% of treated patients with the HCV genotype 1 (Bridge and Sawilowsky, 1999; Brown and

Gaglio, 2003). The recent addition of first-generation protease inhibitors to PEG-IFN/RBV combination therapy improved antiviral efficacy but increased the side effects, especially severe anemia (Kumada et al., 2012; McHutchison et al., 2009a). The increased healthcare costs associated with the addition of protease inhibitors will confine triple therapy usage in many countries with limited sources. Moreover, in patients with favorable predictors for SVR (low pretreatment HCV RNA, IL28 polymorphisms, no advanced fibrosis), dual therapy with PEG-IFN/RBV may still be a choice (Mauss et al, 2012). For the reasons mentioned previously, considering the factors associated with PEG-IFN/RBV combination therapy

outcome is still important for evidence-based treatment recommendations for chronic HCV patients. Sustained viral response (SVR), the primary goal of therapy, is defined as undetectable HCV RNA for at least 24 weeks post treatment (Donlin et al., 2007). Multiple factors including virus-specific characteristics such as viral load, genotype, viral variants within the interferon sensitivity determining region (ISDR), may be responsible for these differences, but also clinical and epidemiological features are connected with a response to the PEG-IFN/RBV combination therapy (Feld and Hoofnagle, 2005; Kau et al., 2008; Wohnsland et al., 2007). Host genetic polymorphisms of several genes such as chemokines, interleukins and interferon-stimulated genes are associated with SVR (Asselah et al., 2007; Kau et al., 2008; Wohnsland et al., 2007). The aim of this study was to identify host and viral factors of response to pegylated interferon-alfa/ribavirin treatment in chronic hepatitis C patients from Serbia, infected with genotype 1b, the most frequent genotype in Serbia (Stamenkovic et al., 2000).

MATERIALS AND METHODS

Patients

Samples were collected from 100 chronic hepatitis C patients before start of treatment with combined antiviral therapy (pegylated interferon-alfa/ribavirin). Samples of patients from Serbia were classified according to the outcome of therapy (50 SVR and 50 NR – non-responders). All patients were registered randomly in the Clinical Center of Serbia and the Military Medical Academy in Belgrade. Patients received pegylated interferon-alfa (180 µg/week) plus ribavirin (800 mg/d) for 24 weeks (HCV genotype 2 and 3) or pegylated interferon-alfa (180 µg/week) plus ribavirin (1000 mg/d, if their body weight was <75 kg, or 1200 mg/d, if it was ≥75 kg) for 48 weeks in patients with HCV genotypes 1 or 4. A sustained virologic response was defined as the absence of HCV RNA (<250 IU/ml) in serum 6 months after the end of treatment. Non-response was defined as the presence of serum HCV RNA (<250 IU/ml) 6 months after the end of treatment. Histological findings were

evaluated according to the stage of fibrosis (F0-F3) and cirrhosis (F4) by METAVIR score. Liver histological staging was based on five degrees of fibrosis as F0 (no fibrosis), F1 (mild fibrosis without septa), F2 (moderate fibrosis with few septa), F3 (severe fibrosis with numerous septa without cirrhosis) and F4 (cirrhosis). These stages were further grouped as F0-F1 (no/minimal fibrosis), F2-F3 (advanced fibrosis) and F4 (cirrhosis). Biochemical analyses were performed at the hospital laboratory by standard procedure. Plasma samples were prepared from the patients before starting therapy. All patients were positive for the antibody to HCV and had a history of raised serum ALTs for more than 6 months. The study was approved by the Ethics Committee of the Faculty of Medicine, University of Belgrade, and all patients provided written consent.

RNA Extraction

Total RNA was extracted from 100 µl of plasma using the Ribo-Sorb-100 (HCV Quant) RNA/DNA Extraction Kit (Sacace biotechnologies, Como, Italy) and dissolved in 50µL of RNA-eluent according to the manufacturer's protocol. The RNA obtained was stored at -20° C until use.

RT-PCR and amplification 5' non translated-region (5'NTR)

The total RNA-HCV was detected by (reverse transcription) RT-PCR. Reverse transcription and first PCR was performed using a QIAGEN One Step RT-PCR Kit (Qiagen, GmbH, Germany), according to the manufacturer's instructions. The selected part of the 5' NTR was amplified by RT-PCR (QIAGEN One Step RT-PCR Kit, Germany) with sense and antisense primers (IR-F *ggcgacactccaccatagat*: IR-R *cacggctctacgagacctccc*), which covered 319 nt. We carried out RT-PCR for 35 cycles (reverse transcription at 50°C for 30 min and at 95°C for 15 min), followed by amplification at 94°C for 45 s, 54°C for 45 s and 72°C for 60 s, with a 7 min final extension at 72°C. The amplified first round PCR products were subjected to two second rounds of PCR amplifications. In the second PCR, the same primers were used as in

RT-PCR with 0.5 U/reaction Dream Taq polymerase (Fermentas, Lithuania). The amplification protocol included initial denaturation for 3 min at 95°C followed by 28 cycles of amplification at 94°C for 45 s, 58°C for 45 s and 72°C for 60 s, with a 7 min final extension at 72°C (thermal cycler Applied Biosystems Gene Amp® PCR System 9700). The final PCR products were analyzed on an 8% polyacrylamide gel stained with silver nitrate and the 319-bp band was confirmed.

Quantitative detection of HCV RNA

The levels of HCV RNA were determined by real-time PCR (Applied Biosystems 7500, Foster City, USA) with the commercially available assay R-TMQ HCV Kit (Sacace Biotechnologies, Como, Italy) according to the manufacturer's instructions.

HCV Genotyping

The genotype of HCV was determined with the (reverse transcription) RT-PCR HCV (HCV Genotype Kit, Sacace Biotechnologies, Como, Italy) according to the manufacturer's instructions (thermal cycler Applied Biosystems Gene Amp® PCR System 9700).

Statistical analysis

Differences in frequency distribution between two or more categorical variables were done by Pearson's χ^2 test. Normality of the continuous variables was tested by Kolmogorov-Smirnov test with Lilliefors' correction. Means of normally distributed continuous variables between sustained virologic responders and non-responders were compared by unpaired t-test and means of skewed continuous variables with nonparametric Mann-Whitney U Test. Results are presented as mean \pm standard deviation (SD). The correlation between ALT levels and RNA viral load was tested by Spearman's rank order correlation test. In all tests, p values of less than 0.05 were considered statistically significant. Statistical analysis was performed using Statistica Version 8.0 software package (StatSoft Inc, 2007) and Microsoft Excel 7.0.

RESULTS

The current study included only patients with HCV genotype 1b divided according to therapy outcome into two groups (50 SVR and 50 NR). The baseline characteristics of the patients overall and divided by response to therapy are shown in Table 1. There were no significant differences between the SVR and NR groups concerning age and gender (Table 1). Both groups had serum ALT levels above the upper limit of normal (>30 U/L), but the ALT level in the NR group was significantly higher than that in the SVR group of patients ($p = 0.046$). Further statistical analysis showed that elevated ALT levels were correlated with viral load (Spearman's $r = 0.21$, $p = 0.04$) (Fig. 1). However, we found no correlation between the stage of liver fibrosis and response to therapy ($p = 0.76$) or baseline clinical features of the patients ($p = 0.57$, data not shown). In our sample, fibrosis progression was independent of genotype, ALT level, pre-treatment viral load and response to therapy. Histologically, 33% of all the patients had moderate fibrosis (F2) and 27% showed evidence of cirrhosis (F4) (Table 1). The effect of pre-treatment viral load on the response to therapy was also studied. Patients with SVR had a slightly lower pre-treatment viral load compared to those with NR, but the difference was not statistically significant (Table 1). Moreover, pre-treatment levels of viral load had no effect on liver histology ($p = 0.93$, data not shown). The major route of HCV transmission among our patients overall was unknown (45%), followed by blood transfusion (25%), intravenous drug use (IVDU) (18%), previous surgery (7%), hemodialysis (4%) and high risk professional activity (1%) (Table 1). There were significant differences between the groups of patients concerning route of HCV transmission and response to antiviral therapy ($p = 0.02$). With regard to response, the unknown route of HCV transmission and transmission by IVDU were significantly more frequent in the patients exhibiting SVR than among the non-responders. On the other hand, a history of HCV transmission by blood product transfusion and hemodialysis were significantly more common in NR patients than in those with SVR (Table 1). In addition, intravenous drug users were significantly

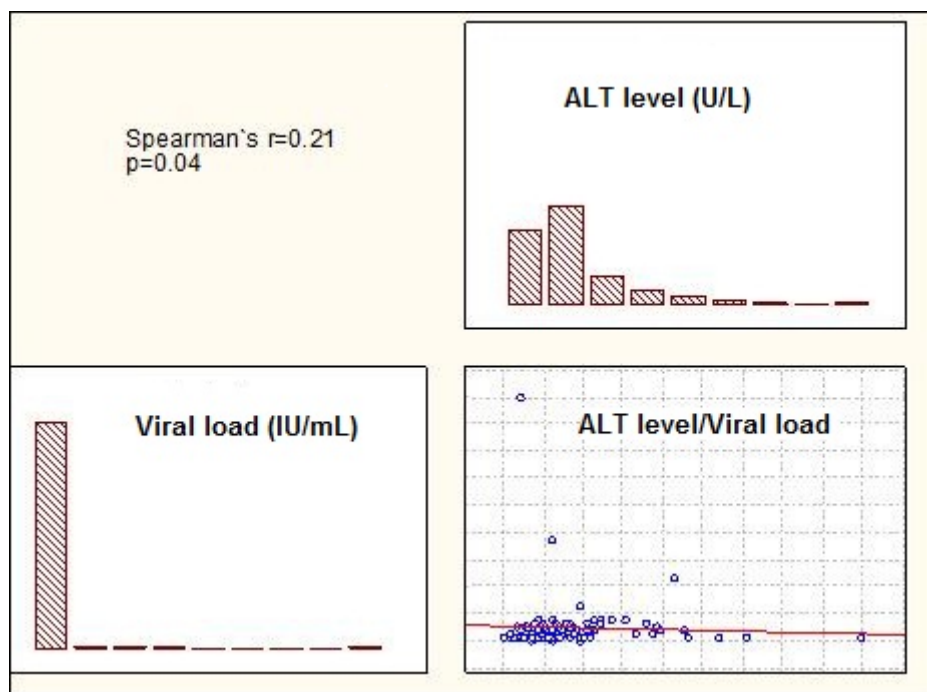


Fig. 1. Spearman correlation of ALT levels and viral load in HCV patients. Elevated levels of serum ALT were significantly associated with pre-treatment viral load ($p < 0.05$).

younger than patients with a history of blood transfusion and those with an unknown route of HCV transmission (mean age \pm SD: 33.6 ± 7.4 vs. 42.6 ± 12.3 vs. 45.0 ± 11.8 , respectively, ANCOVA post-hoc LSD test $p = 0.03$, adjusted for gender). With regard to gender, men were significantly more common in the group of intravenous drug users than women (83% vs. 17%, $p < 0.0001$).

DISCUSSION

Although HCV infection resolves spontaneously in some patients, about 85% of them develop chronic infection and represent potential candidates for antiviral therapy (Marcellin, 1999; Liang et al., 2000). To predict the treatment response before starting antiviral treatment it is important to consider baseline factors associated with SVR to PEG-IFN/RBV, such as HCV genotype, degree of liver fibrosis, pre-treatment viral load, age, gender and ethnicity. According to current recommendations, HCV genotype and pre-treatment viral load are the only parameters rel-

evant for an estimation of treatment duration (Ghany et al., 2009; McCaughan et al., 2007; Zeuzem et al., 2009). There is a wealth of evidence for age as an important host factor in the treatment response. Some large prospective studies showed that age correlated significantly with SVR and patients younger than 40–45 years had the best response rates (Fried et al., 2002; Manns et al., 2001; Poynard et al., 2000). Considering the above-mentioned, we observed that younger patients had slighter higher response rates to antiviral therapy than older ones, but the difference was not statistically significant, which confirms earlier findings (Fried et al., 2002; Hadziyannis et al., 2004; Manns et al., 2001; McHutchison et al., 2009b; Zeuzem et al., 2004). It has been demonstrated that patients with normal ALT levels have a similar virologic response to interferon therapy as patients with elevated ALT levels (Gordon et al., 2000; Rossini et al., 1997; Van Thiel et al., 1995). In contrast, we found that ALT level was significantly higher in NR than in SVR patients. Both groups of patients had serum ALT above the upper limit of normal, and one

Table 1. Baseline clinical features of HCV genotype 1b patients according to response to therapy.

Clinical and pathological characteristics of patients	Sustained responders (n = 50)	Non-responders (n = 50)	HCV patients overall Value \pm SD	P
Age (years) ^a	40.44 \pm 11.78	43.74 \pm 12.31	42.09 \pm 12.10	ns*
Sex, n (female/male)	22/28	22/28	44/56	ns
ALT level (U/L) ^a	80.88 \pm 56.88	92.62 \pm 46.54	86.75 \pm 52.04	0.046 [#]
HCV RNA level (IU/mL) ^c	463522.0 \pm 662955.7	749411.2 \pm 1635497.7	606466.6 \pm 1249837.0	ns [#]
Transmission of HCV infection	n (%)	n (%)	All n (%)	
Unknown	27 (54)	18 (36)	45 (45)	<0.005
Post-transfusion	8 (16)	17 (34)	25 (25)	<0.005
Hemodialysis	0	4 (8)	4 (4)	<0.005
Intravenous drug use	12 (24)	6 (12)	18 (18)	<0.025
Previous surgery	2 (4)	5 (10)	7 (7)	ns
High risk professional activity	1 (2)	0	1 (1)	ns
Stage of fibrosis ^b	n (%)	n (%)	All n (%)	
F0	3 (6)	4 (8)	7 (7)	ns
F1	9 (18)	9 (18)	18 (18)	ns
F2	19 (38)	14 (28)	33 (33)	ns
F3	8 (16)	7 (14)	15 (15)	ns
F4	11 (22)	16 (32)	27 (27)	ns

^a- data expressed as mean \pm SD; ^b- stage of fibrosis expressed by METAVIR score (fibrosis 0, 1, 2, 3, and cirrhosis 4); ^c- expressed HCV RNA level $\times 10^5$ IU/mL; comparison was done between sustained responders and non-responders: **p** - for categorical variables Pearson Chi square test was used; * - comparison of normally distributed continuous variables between sustained responders and non-responders using Students' t-test; [#]-comparison of skew distributed continuous variables between sustained responders and non-responders by Mann-Whitney U test; -p- values < 0.05 were considered statistically significant; ns-not statistically significant.

possible explanation is that younger patients were enrolled in our study and higher ALT levels were reported to be associated with an active host immune response to viral populations and rapid elimination of the infected hepatocytes (Bozdayia et al., 2000; Gavazzi and Krause, 2002; Gordon et al., 2000; Uto et al., 2012). An elevated ALT level acts as an indicator of hepatocyte injury (Murakami et al., 2004; Ong et al., 1999). We found no significant difference between increased serum ALT and stage of liver fibro-

sis ($p = 0.93$), which is in line with the observation of Puoti and colleagues (1997), while Zechini et al. (1994) detected a correlation between serum ALT level and liver damage. With respect to virus-related factors, HCV pre-treatment viral load has been suggested as a predictor of SVR (Ijaz et al., 2011; Manns et al., 2001; Salmerón et al., 2008). In addition, only the pre-treatment viral load was observed to be essential for successful standard antiviral therapies (Berg et al., 2000; Fried et al., 2002; Izumi et al., 2010;

Manns et al., 2001). In our study, average viral loads were higher in non-responders, but differences were not significant, which is in accordance with earlier results (Araújo et al., 2002; Idrees et al., 2009; Izumi et al., 2010; Kanai, 1992; Lau, 1993; Shiffman et al., 2007; Zuberi et al., 2008). We found no statistically significant differences between pre-treatment viral load and stage of liver fibrosis, which confirms some previous results (Kao et al., 1996; Zeuzem et al., 1996), but does not comply with the finding of a significant correlation between serum HCV RNA titer and liver damage (Fanning et al., 1999). While some authors noted no evidence of correlation between serum viral load and ALT (Fanning et al., 1999; Liu et al., 2009), they did find a significant association with the rate of high ALT levels (ALT>40U/L), which is in line with our observation of a high average ALT concentration. In agreement with others, the current study revealed a significant difference between route of HCV transmission and age (Cornberg et al., 2011; Gheorghe et al., 2008; Nemecek and Strunecky, 2009). We also investigated the relationship between route of transmission and sex. We found that men were more frequent in the group of intravenous drug users than women were, but among patients with a positive history blood transfusion, there were considerably more women than men, which is in accordance with the large epidemiological study of Cornberg et al. (2011). Moreover, we found significant differences between several routes of HCV transmission and response to therapy. Unknown routes of HCV transmission and transmission by intravenous drug use were significantly more frequent in the SVR than in NR patients. On the other hand, a history of blood product transfusion and the hemodialysis route of HCV transmission were more common in NR than in SVR patients. These findings may suggest that infectious dose and route of infection could be connected with different responses to therapy. In the Serbian population, the unknown route of transmission is dominant (Cornberg et al., 2011). It has been suggested that the high proportion of cases with an unknown transmission route could be partly due to delay between infection and diagnosis, or the result from other common exposures (such as nosocomial transmission); it may also reflect the individual's

desire not to disclose delicate information (Chaves et al., 2003). Other significant risk factors for HCV transmission in the population of Serbia were transfusion and intravenous drug use. Similar observations were obtained in some earlier epidemiological studies (Alter, 2007; Cornberg et al., 2011). Some host factors previously associated with treatment failure, such as advanced age, advanced liver fibrosis stage and cirrhosis, did not reach statistical significance in our study. These discrepant results may be due to one or more factors. The first is probably related to the case control study design matched for age, sex, genotype and response to therapy. The second may be connected to the relatively small number of enrolled patients. However, the present investigation has demonstrated that pre-treatment viral load, ALT level and route of HCV transmission are important factors in the response to therapy, but should not be used alone to predict disease outcome. Further research ought to include comparative analysis of human and viral genome specificity with special focus on the polymorphism of genes participating in the human immune response, such as IL28B.

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