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Non-structural 3 protein expression is associated with T cell protein tyrosine phosphatase and viral RNA levels in chronic hepatitis C patients

Nogol Rahbin [a,b], Lars Frelin [a], Soo Aleman [b], Rolf Hultcrantz [b], Matti Sällberg [a], Erwin Daniel Brenndörfer [a,*]

[a] Division of Clinical Microbiology, Department of Laboratory Medicine, Karolinska Institutet at Karolinska University Hospital Huddinge, 14186 Stockholm, Sweden
[b] Department of Gastroenterology and Hepatology, Karolinska University Hospital Solna and Huddinge, 17176 Stockholm, Sweden

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ABSTRACT

The hepatitis C virus (HCV) non-structural 3 (NS3) protein plays key roles in both the viral life cycle and in the modulation of intrahepatic signaling and immunity. We recently showed that NS3 cleaves the T cell protein tyrosine phosphatase (TCPTP).

To better understand the inactivation of TCPTP in HCV-infected humans, we investigated whether there is an association between TCPTP cleavage, NS3 protein levels and clinical parameters in hepatitis C patients.

Liver biopsies were obtained from 69 HCV RNA positive patients with confirmed chronic HCV infection and 16 control patients. Hepatic NS3 and TCPTP protein levels were determined and correlated to viral load or clinical parameters for the severity of liver disease.

We found a positive correlation between the viral load and the intrahepatic NS3 protein levels in patients infected with HCV. HCV-infected patients had significantly lower intrahepatic TCPTP levels than non-infected control patients. In HCV-infected patients both intrahepatic NS3 expression and the viral load were inversely correlated with the intrahepatic TCPTP protein levels. Detection of NS3 did not associate with any other clinical parameters such as liver damage, the grade of liver inflammation or fibrosis stage.

This is the first study reporting a detailed analysis of HCV NS3 and TCPTP protein levels in the liver. It demonstrates a clear link between HCV viral load, NS3 expression in the liver and intrahepatic TCPTP levels. Thus, the association between TCPTP cleavage and viral replication may have important consequences for the HCV life cycle and HCV-induced liver diseases.

1. Introduction

Worldwide there are about 170 million patients infected with hepatitis C virus (HCV). Eighty percent of the patients exposed to HCV develop a chronic infection, which may result in liver fibrosis, liver cirrhosis and/or hepatocellular carcinoma [1,2]. The high capability of HCV to establish chronicity and to evade interferon-α (IFNα)/ribavirin-based treatment shows that HCV has evolved sophisticated escape strategies for both the innate and the adaptive immune system [3,4]. Thus, a better understanding of the mechanisms resulting in HCV persistence is an urgent need.

The HCV genome codes for a polyprotein, which is co- and post-translationally cleaved in 3 structural (core, E1, E2) and 7 non-structural (NS) proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) [5]. The HCV NS3/4A protein complex combines the enzymatic activities of a protease, which is needed for polyprotein cleavage and of a helicase, which is responsible for unwinding and separation of the replicating double-stranded RNA. However, NS3/4A is not only essential for the viral life cycle but also known to modulate hepatic signaling pathways. NS3/4A is able to block the cellular IFN response to double-stranded RNA through both abrogation of the retinoic acid-inducible gene-I (RIG-I)-mediated pathway by cleaving mitochondrial antiviral signaling protein (MAVS) [6,7] and inactivation of TOLL-like receptor 3-mediated signaling by cleaving Toll/IL-1 receptor domain-containing adaptor inducing IFNβ (TRIF) [8]. Furthermore, intrahepatic expression of NS3/4A results in an improvement of hepatocyte survival and liver

Abbreviations: HCV, hepatitis C virus; NS, non-structural; TCPTP, T cell protein tyrosine phosphatase; IFN, interferon; EGFR, epidermal growth factor receptor; Jak, janus kinase; STAT, signal transducer and activator of transcription; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IgG, immunoglobulin G; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase.

* Corresponding author. Address: Department of Laboratory Medicine, Division of Clinical Microbiology F68, Karolinska Institutet at Karolinska University Hospital Huddinge, S-141 86 Stockholm, Sweden. Fax: +46 8 58587933.
E-mail address: erwin.brenndorfer@ki.se (E.D. Brenndörfer).

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regeneration by enhancing nuclear factor κB activation and increasing the intrahepatic levels of CCL2 and tumor necrosis factor-α [9]. Additionally, NS3/4A interferes with intrahepatic immunity by modulating the expression of the chemokines CXCL9 and CCL17 causing a shift of the intrahepatic T-helper cell 1 (Th1)/Th2 balance towards a Th2 response [10].

We recently discovered that NS3/4A cleaves the T cell protein tyrosine phosphatase (TCPTP) thereby disrupting an important negative feedback regulation of epidermal growth factor receptor (EGFR) signaling and Akt activation [11]. This is of particular importance when the role of EGFR activity for HCV cell entry [12] and the implication of EGFR and Akt activation in the development of hepatocellular carcinoma [13,14] are taken into consideration. Furthermore, TCPTP may influence intrahepatic immunity by regulating the janus kinase/signal transducer and activator of transcription (Jak/STAT) pathway.

The intrahepatic NS3 and TCPTP protein levels have not been studied in detail in liver biopsies from HCV-infected patients. We therefore performed a thorough analysis of their protein levels in the liver by western blot analysis and correlated these with virological and clinical markers.

2. Materials and methods

2.1. Patients and clinical data

Liver tissue was obtained from patients undergoing biopsy for staging of liver disease after oral and written informed consent and with approval from the Regional Ethics Committee in Stockholm according to the ethical guidelines of the 1975 Declaration of Helsinki. Liver inflammation (grade 0–4) and liver fibrosis (stage 0–4) have been assessed with the Metavir grading algorithm. High alcohol consumption was defined as $\geqslant$50 g/day ethanol for males and $\geqslant$40 g/day for females.

2.2. Immunoprecipitation and Western blot analysis

Liver biopsies from chronically HCV-infected or control patients were homogenised and analysed by immunoprecipitation followed by Western blot (NS3) or by western blot (TCPTP and GAPDH). In brief, 5 mg of each biopsy was lysed in 1 ml buffer (150 mM NaCl, 50 mM Tris/HCl pH 7.4, 1% Triton-X 100, 1% Na-deoxycholate, 1% sodium dodecyl sulphate (SDS), 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol and 1 mM Na_3VO_4), homogenized and sonicated twice for 30 s. For NS3 detection, protein A Sepharose and anti-NS3 mouse polyclonal antibody (cross-reactive, in-house produced) were added and incubated overnight at 4 °C. The washed pellets were re-suspended in SDS sample buffer, heated at 98 °C for 5 min prior to SDS–PAGE on 4–12% Bis–Tris gels (Invitrogen, Paisley, UK) and transferred to Nitrocellulose membranes. Nonspecific binding was blocked with 5% (w/v) non-fat dry milk powder in phosphate buffered saline (PBS)-T (20 mM Tris/HCl pH 7.4, 137 mM NaCl, and 0.05% Tween) or 5% bovine serum albumin (BSA) for 1 h at room temperature. The blots were incubated overnight in PBS-T supplemented with primary antibodies. After extensive rinsing with PBS-T, blots were incubated with secondary antibody (goat anti-mouse IgG) conjugated to horseradish peroxidase for 1 h (Dako, Glostrup, Denmark). After further rinsing in PBS-T, the immunoblots were developed with the enhanced chemiluminescence system (ECL; PerkinElmer, Shelton, CT, USA) following the manufacturer's instructions. NS3 was detected by using the anti-NS3 mouse polyclonal antibody. As positive controls, we used lysates from HepG2 cells (American Type Culture Collection, Manassas, VA, USA) transfected with plasmids coding for NS3/4A. As negative controls, homogenates of liver biopsies from non-HCV-infected patients were used. For TCPTP and GAPDH detection, 30–40 μg of protein/lane were used for SDS–PAGE analysis. Protein concentration was estimated by using the BioRad protein assay (BioRad Laboratories, Hercules, CA, USA). Antibodies against TCPTP and GAPDH were obtained from R&D Systems (Minneapolis, MN, USA) and Biodesign (Saco, ME, USA), respectively.

2.3. Statistical analysis

The densitometrical analysis of the bands obtained through Western blot analysis was performed by using ImageJ. The values for each group were compared using the Mann–Whitney *U* test by aid of the InStat 3 software. The correlations after the Spearman's approach were determined by using the GraphPad Prism software.

3. Results

3.1. Patient characteristics

A total of 69 patients with chronic hepatitis C were included in this study. Of these, 37 (53.6%) were male and 32 (46.4%) female. The majority of the patients (n = 37; 53.6%) were infected with the HCV genotype 1. Intravenous drug abuse was the major route of infection constituting 36.2%. The age of the study participants at the time of the biopsy was 50.7 ± 10.4 years (Table 1). The control group consisted of 16 patients (8 male and 8 female patients) with an age of 44.6 ± 11.0 years. The control patients were diagnosed with alcohol-induced hepatitis (37.5%), hepatitis B (31.3%), unspecific chronic active hepatitis (18.8%), hemochromatosis (6.2%) and non-alcoholic steatohepatitis (6.2%).

3.2. NS3 detection and correlation to clinical markers

The NS3 protein is a key viral enzyme with activities as both protease and helicase. NS3 is not only essential for the viral life cycle but also known to modulate hepatic signaling pathways by cleaving transduction molecules. Thus, we decided to perform a thorough analysis of NS3 expression in HCV-infected patients and to correlate NS3 levels with clinical and virological markers and intrahepatic TCPTP levels. The detection of intrahepatic NS3 protein was performed by immunoprecipitation followed by Western blot analysis (Fig. 1A). NS3 was detected in 31 of 69 liver samples (44.9%), and was more commonly detected in patients infected with the HCV genotype 1 than in those with non-1 genotypes (56.8% versus 28.6%) (Table 1).

To analyse if the presence of detectable NS3 protein was correlated to liver injury, mean serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were compared (Table 1). However, no significant differences between NS3-positive and NS3-negative patients were found. Correspondingly, detection of NS3 was not statistically associated with the grade of liver inflammation or the fibrosis stage (Table 1).

From the 50 patients we obtained data about their alcohol consumption, 24 were NS3-positive. Interestingly, there was a trend of NS3-positivity in patients with a history of high alcohol consumption with NS3 detected in 7/9 (77.8%) of these patient samples as compared to 17/41 NS3-positive samples (41.5%) in patients without such history (p = 0.069; Fischer's exact test) (Table 1).

3.3. Correlation between intrahepatic NS3 levels and viral load

One could hypothesize that a higher viral load would result in a higher number of HCV-infected hepatocytes and a higher

Table 1
Characteristics of the chronically HCV-infected patients.

	HCV-infected patients (%)	NS3-positive HCV-infected patients (%)	NS3-negative HCV-infected patients (%)
Number of patients	69 (100.0)	31 (44.9)	38 (55.1)
Gender			
Male	37 (53.6)	19 (51.4)	18 (48.6)
Female	32 (46.4)	12 (37.5)	20 (62.5)
Age	50.7 ± 10.4	50.0 ± 8.0	51.3 ± 12.1
Viral load* (10^6 IU/ml)	2.41 ± 4.04	3.81 ± 4.94	1.38 ± 2.92
HCV genotype			
1	37 (53.6)	21 (67.7)	16 (42.1)
2	12 (17.4)	5 (16.1)	7 (18.4)
3	12 (17.4)	3 (9.7)	9 (23.7)
4	4 (5.8)	0 (0.0)	4 (10.5)
Unknown	4 (5.8)	2 (6.5)	2 (5.3)
Potential routes of infection			
Intravenous drug abuse	25 (36.2)	14 (45.2)	11 (28.9)
Blood transfusion	17 (24.6)	7 (22.6)	10 (26.3)
Other (i.e. tattoo, sex)	8 (11.6)	1 (3.2)	7 (18.4)
Unknown	19 (27.5)	9 (29.0)	10 (26.3)
ALT (microkat/l)	1.66 ± 1.44	1.48 ± 1.45	1.80 ± 1.44
AST (microkat/l)	1.12 ± 0.75	1.01 ± 0.73	1.21 ± 0.77
ALP (microkat/l)	1.20 ± 0.35	1.22 ± 0.37	1.18 ± 0.35
Grade of liver inflammation	1.49 ± 0.65	1.48 ± 0.65	1.50 ± 0.66
Fibrosis stage	1.64 ± 1.03	1.56 ± 1.08	1.68 ± 1.00
High alcohol consumption	9 (18.0)	7 (29.2)	2 (7.7)

HCV = hepatitis C virus; NS = non-structural; ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase.
*The viral load at the time of the liver biopsy was only known from 52 of the 69 patients (22 of 31 NS3-positive and 30 of 38 NS3-negative HCV patients).

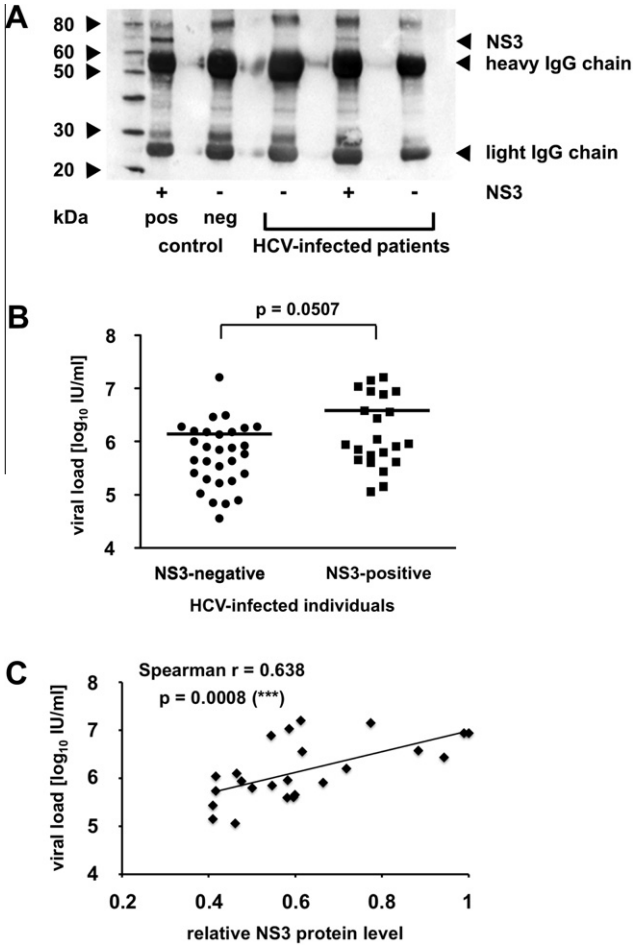

Fig. 1. Detection of NS3 protein in liver biopsies from HCV-infected patients and correlation with the viral load. (A) Liver lysates from HCV-infected patients as well as a positive (lysates from HepG2 cells transfected with plasmids coding for NS3/4A) and a negative (homogenates of liver biopsies from non-HCV-infected patients) control sample were analyzed for NS3 in immunoprecipitation followed by Western blot. A representative example is shown. (B) HCV patients negative (left) or positive (right) for NS3 in immunoprecipitation followed by Western blot are plotted against the HCV viral load. The viral load at the time of the liver biopsy was known from 52 of the 69 patients. (C) Correlation of HCV viral load and the relative NS3 protein levels. NS3 protein expression was analyzed by immunoprecipitation followed by Western blot. The relative NS3 protein levels represent the ratio of the net intensity of the NS3 band and the light IgG chain band. The highest value was set to 1. The viral load at the time of the liver biopsy was known from 24 of the 31 NS3-positive patients.

intrahepatic expression of HCV NS3. In fact, patients with detectable NS3 had mean HCV RNA levels of 3.81×10^6 IU/ml ($\pm 4.94 \times 10^6$ IU/ml) as compared to 1.38×10^6 IU/ml ($\pm 2.92 \times 10^6$ IU/ml) in patients without detectable NS3. Thus, there was a trend towards higher viral load in patients with detectable NS3 ($p = 0.0507$; Mann Whitney U test) (Table 1, Fig. 1B). Furthermore, it was more common that NS3-positive HCV-infected patients had very high levels ($>2 \times 10^6$ IU/ml) of serum HCV RNA as compared to NS3-negative HCV-infected patients (9/22 versus 3/30 in NS3-negative patients; $p = 0.0175$; Fischer's exact test).

To analyze the correlation of intrahepatic NS3 levels with the viral load more in detail, we performed a densitometry analysis of the NS3 bands obtained by Western blot. The measured relative NS3 expression levels showed a significant correlation to the viral load of the respective patients (Spearman r of 0.638 and $p = 0.0008$) (Fig. 1C).

Overall, the data reveal that a high viral load indeed results in high intrahepatic HCV NS3 levels and vice versa.

3.4. Correlation between intrahepatic NS3 expression and TCPTP levels

We previously reported the discovery that the ubiquitously expressed phosphatase TCPTP is a novel substrate of the HCV NS3/4A protease [11]. However, a detailed quantitative and qualitative analysis of intrahepatic TCPTP levels and their relation to HCV RNA and NS3 protein levels in HCV patients has not been performed. We therefore compared these parameters in liver biopsies from 16 NS3-positive HCV patients and 16 control patients not infected with HCV (Fig. 2). We found significantly lower intrahepatic levels of TCPTP in HCV patients compared to control patients ($p < 0.001$; Mann Whitney U test). Since TCPTP is a direct substrate of the NS3/4A protease, we investigated whether a correlation between intrahepatic TCPTP and NS3 levels exists. We found a significant inverse correlation between intrahepatic TCPTP and NS3 protein levels (Spearman r of -0.571 and $p = 0.021$), suggesting that the more NS3 is present in the liver the more TCPTP is cleaved resulting in lower total intrahepatic TCPTP levels (Fig. 3A).

3.5. Correlation between intrahepatic TCPTP levels and viral load

Since we could demonstrate that the intrahepatic NS3 levels were correlated to the viral load, we investigated if any relation exists between intrahepatic TCPTP levels and the viral load. We found an inverse correlation between intrahepatic TCPTP levels and the viral load in patients infected with HCV (Spearman r of -0.741 and $p = 0.008$) (Fig. 3B). This suggests that the inactivation of TCPTP may serve as a strategy of HCV to modulate signaling pathways. In the infected liver the cleavage of the phosphatase TCPTP, known to be involved in the negative regulation of EGFR, Akt and Jak/STAT signaling, may favor viral persistence.

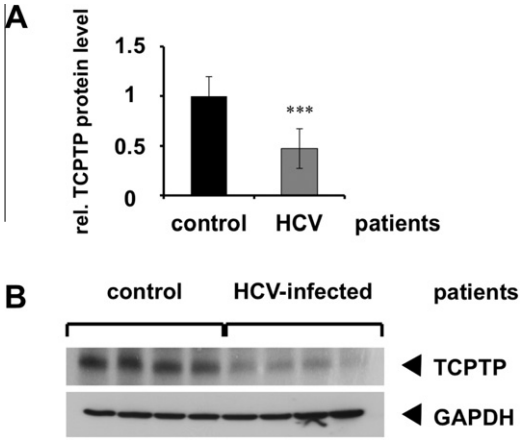

Fig. 2. Intrahepatic TCPTP levels in liver biopsies from chronically HCV-infected and control patients. (A) Total protein extracts were prepared from liver biopsies and analyzed by Western blot for TCPTP and GAPDH (loading control). The relative TCPTP protein levels represent the ratio of the net intensity of the TCPTP band and the GAPDH band. The ratio representing the control patients was set to 1. (B) A representative example of a Western blot for TCPTP and GAPDH is shown.

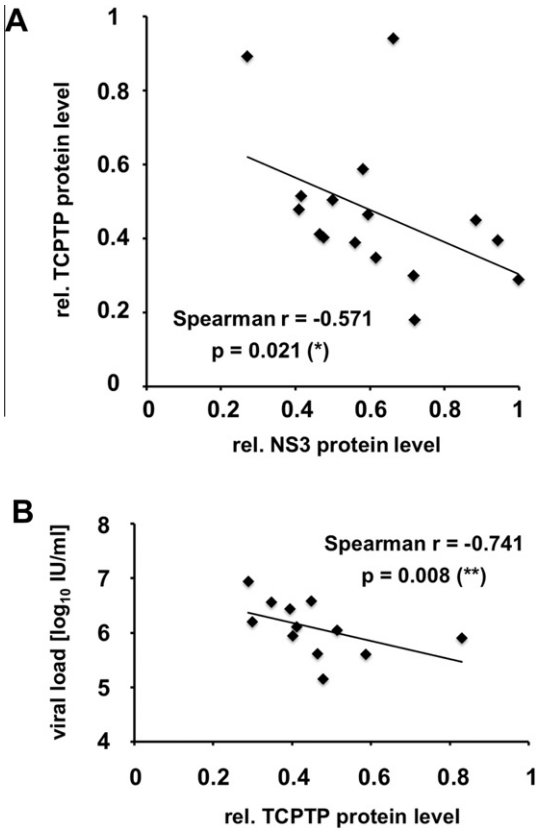

Fig. 3. Correlation of intrahepatic TCPTP levels with intrahepatic NS3 levels and viral load. (A) Correlation of TCPTP and NS3 levels in liver biopsies from chronically HCV-infected patients. Total protein extracts were prepared from liver biopsies and analyzed by Western blot for TCPTP and GAPDH (loading control). The relative TCPTP protein levels represent the ratio of the net intensity of the TCPTP band and the GAPDH band. NS3 protein expression was analyzed by immunoprecipitation followed by Western blot. The relative NS3 protein levels represent the ratio of the net intensity of the NS3 band and the light IgG chain band. (B) Correlation of HCV viral load and the relative intrahepatic TCPTP protein levels in chronically HCV-infected patients. The viral load at the time of the liver biopsy was only known from 13 of the 16 analyzed patients.

4. Discussion

The hepatitis C virus is a hepatotropic virus resulting in chronic infection of the liver in a majority of the infected patients. Even though HCV produces daily up to 10^{12} new virions during chronic infection [15], detection of HCV proteins in the liver of hepatitis C patients has proven to be difficult [16–19], suggesting that HCV proteins are expressed only at low levels. Furthermore, only 1.7–21.6% of hepatocytes in chronic HCV patients are infected by HCV [20]. This study is to our knowledge the first report demonstrating that the HCV NS3 protein can be detected in liver tissue from HCV-infected patients by a combination of immunoprecipitation and Western blot analysis. This allows for studies on the association between intrahepatic NS3 levels with clinical parameters, virological markers and signaling pathways modulated by HCV and/or NS3. NS3 could be detected in 45% of the liver biopsies from chronic hepatitis C patients, and NS3-positivity was more common when these had very high levels ($>2 \times 10^6$ IU/ml) of serum HCV RNA. Furthermore, detection of NS3 was more common in patients with the HCV genotype 1 than in patients infected with non-genotype 1 HCV. This may be related to the increased ratio of genotype 1 patients with a high viral load. The cross-reactivity of the used antibody was confirmed by the fact that NS3 was also detected in genotype 2 and 3 patients.

We could not find an association of NS3 detection with the blood levels of liver damage markers, the grade of liver inflammation or the fibrosis stage. These findings imply that liver injury caused by HCV infection is not directly proportional to the viral load or the expression level of HCV proteins. In agreement with our data, a number of other studies could also not find a correlation between the serum titers of HCV and severity of liver disease [21–23].

An unexpected observation was that there seemed to be a trend of a more frequent detection of NS3 in patients with a history of high alcohol consumption, suggesting that alcohol may have a positive effect on HCV replication. The possible influence of alcohol on HCV viral replication has been analyzed by a number of studies with variable results. While some studies describe increased HCV RNA levels in alcoholics [24,25], others have found no difference between drinkers and abstinent individuals [26,27]. Overall, we have no good explanation for this observation.

We found a clear correlation between intrahepatic NS3 protein levels and the HCV viral load. This suggests that a higher viral load is a consequence of a higher number of HCV-infected hepatocytes and a higher amount of translated HCV proteins.

Patients chronically infected with HCV have significantly lower intrahepatic levels of TCPTP than control patients. Importantly, we found an inverse correlation between intrahepatic NS3 protein levels and intrahepatic levels of the tyrosine phosphatase TCPTP. Consistent with this, we found an inverse correlation between the viral load and intrahepatic TCPTP levels. This suggests that a high viral load results in an increased number of HCV-infected hepatocytes producing HCV NS3/4A in which TCPTP is cleaved causing an overall decrease in intrahepatic TCPTP protein levels. The remaining TCPTP may be present in liver cells not infected by HCV and thus not expressing NS3/4A.

The fact that TCPTP-deficient mice die 3–5 weeks after birth because of systemic inflammation [28] shows the fundamental role of TCPTP as negative regulator of diverse signal transduction pathways. By regulating the Jak/STAT pathway [29–32], TCPTP is able to influence both hematopoiesis and the immune response. In addition, TCPTP plays important roles in the control of cellular proliferation by modulating EGFR and PDGFR activity [33] and of glucose homeostasis by dephosphorylating the insulin receptor [34].

The NS3/4A-mediated cleavage of TCPTP may have important implications for the HCV life cycle and the development of HCV-induced liver pathologies. An increase in EGFR activity enhances both HCV replication [11] and HCV cell entry [12]. Furthermore, TCPTP cleavage may cause perturbations of the intrahepatic immune response hence contributing to HCV persistence. Moreover, changes in EGFR and Akt activation induced by TCPTP cleavage may be involved in the development of hepatocellular carcinoma [13,14].

In conclusion, our data demonstrate a significant correlation between the HCV viral load, intrahepatic NS3 and TCPTP protein levels. A high viral load is associated with high intrahepatic NS3 levels and low intrahepatic TCPTP levels. This may have important implications for the HCV life cycle and HCV-induced liver diseases as well as intrahepatic immunity and signal transduction.

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