Tissue Expression of the Hepatitis C Virus NS3 Protein Does Not Correlate with Histological or Clinical Features in Patients with Chronic Hepatitis C

Wei-Hsuan Liao, MD; Shui-Yi Tung, MD; Cheng-Han Shen, MD; Kam-Fai Lee', MD; Cheng-Shyong Wu, MD

Background: In chronic hepatitis B, the HBcAg viral protein in liver tissue demonstrates a positive correlation with serum aminotransferase levels, serum hepatitis B viral DNA, and histological activities. Little is known if similar relationships exist for chronic hepatitis C. This study attempted to determine if expression of the hepatocyte NS3 protein of the hepatitis C virus (HCV-NS3) was correlated with the serum HCV-RNA load, hepatitis activity, or other clinical parameters.

Methods: Clinical and histological data of 214 patients with chronic hepatitis C were retrospectively reviewed. A mouse monoclonal antibody was used to detect HCV-NS3 in hepatocytes. The staining intensity was scored semiquantitatively as 0~3+, and its correlations with the serum HCV-RNA load, hepatitis activity, and other clinical parameters were analyzed.

Results: In total, 202 (94%) of the 214 liver biopsies were positive for HCV-NS3, and the intensity of HCV-NS3 staining was 0 in 12 (6%), 1+ in 181 (84%), and 2+ in 21 patients (10%). The intensity of HCV-NS3 expression in the samples did not correlate with patient age (p = 0.302, ANOVA), patient gender (p = 0.130, Fisher’s exact test), the alanine transaminase level (p = 0.177, ANOVA), serum HCV-RNA level (p = 0.305, ANOVA), HCV antibody titer (p = 0.139, Chi-squared test), hepatitis activity index score (p = 0.861, Chi-squared test), or sustained viral response rate (p = 0.861, Chi-squared test).

Conclusions: This HCV-NS3 immunohistochemical staining method was reliable for detecting HCV in liver specimens. Hepatocyte expression of HCV-NS3 was not correlated with the serum viral load, severity of hepatic injury, or treatment response.

(Chang Gung Med J 2011;34:260-7)

Key words: HCV-NS3 antigen, immunohistochemistry, HAI score
be 4.4% among the general population. Although serological and molecular biological tests are commercially available, a simple and reproducible immunohistochemical (IHC) method that identifies and localizes HCV in normal and pathological liver tissues from humans is still lacking. Previous studies published IHC protocols for HCV detection in liver tissues with good concordance with reverse-transcription polymerase chain reaction (RT-PCR)-based methods, despite the high variability of IHC sensitivity compared to the RT-PCR. The development of mouse monoclonal antibodies to the recombinant NS3 protein of HCV make it possible to observe virus-specific staining in the cytoplasm of hepatocytes with acute HCV infections and in immunosuppressed patients with chronic HCV infection. The biological role of the nonstructural NS3 protein remains unknown, although some data indicated that the core, NS3, and NS5A proteins promote cell growth via modulation of cell-cycle regulator genes.

An IHC analysis of the HBcAg viral protein in the liver during chronic hepatitis B virus (HBV) infection revealed a positive correlation among serum aminotransferase levels, serum HBV DNA loads, and histological activities. Hence, detection of this HBV epitope can serve as a marker of HBV replication. Similar observations for HCV were discussed in a number of studies; however, the conclusions are controversial. Kasprak et al. showed that the tissue expression of HCV-NS3 did not correlate with the histologic grade or stage in patients with chronic hepatitis C. However, the case number in that previous study was small, and the sensitivity of the IHC method used was limited. As such, the strength of their conclusions might have been limited by these biases. The present study attempted to determine if there were correlations among hepatocyte HCV-NS3 expression, the serum HCV-RNA load, hepatitis activity, and other clinical parameters by employing a large case study and a sensitive IHC method.

**METHODS**

In total, 513 consecutive patients with chronic hepatitis C were followed at Chang Gung Memorial Hospital, Chiayi, Taiwan, between January 2003 and December 2008. Chronic hepatitis C was defined as being seropositive for antibodies to HCV for more than 6 months based on an enzyme-linked immunosorbent assay (ELISA) (AxSYM HCV vers. 3.0; Abbott, Ludwigshafen, Germany). Medical records of these patients were reviewed, and information on demographic data, hematology, biochemistry, serology, hepatitis markers, HCV genotypes (INNO LiPa HCV II assay; Innogenetics, Zwijnaarde, Belgium), HCV-RNA levels (Amplicor HCV test vers. 2.0 with a sensitivity of 600 IU/mL; Roche, Mannheim, Germany), and liver biopsy histological examinations (hepatitis activity index (HAI) scores) was retrospectively reviewed. Patients with the following conditions were excluded: (1) positive for the hepatitis B surface antigen (HBsAg) or HIV antibodies; (2) evidence of decompensated liver cirrhosis or hepatocellular carcinoma; (3) past antiviral treatment; (4) a known history or serological evidence of autoimmune liver disease, inheritable disorders such as hemochromatosis, or Wilson’s disease; (5) excessive alcohol intake (daily alcohol consumption exceeding 30 g) or drug abuse, or (6) no liver biopsy specimen. Six patients with chronic hepatitis B who did not have chronic hepatitis C were enrolled as a control group (HBsAg [+], HCV [-]). Among these 6 patients, 2 had serum alanine transaminase (ALT) levels < 2-fold that of the baseline, 2 had ALT levels of 2~5-fold that of the baseline, and 2 had ALT levels > 5-fold that of the baseline.

**Liver biopsy specimens**

Transcutaneous needle liver biopsy specimens were collected from treatment-naïve patients with chronic hepatitis C after informed consent was obtained. They were fully informed of the nature of the disease and the diagnostic procedures involved. The length and diameter of the puncture needles were 1.0~3.5 cm and 2~3 mm, respectively. Specimens were then fixed in 10% formaldehyde and embedded in paraffin. After routine tissue processing, a histological diagnosis was made on hematoxylin and eosin-stained sections. The severity of hepatitis and liver fibrosis were evaluated using an HAI system proposed by Knodell et al.

**Immunohistochemical staining and semiquantification of the HCV-NS3 protein**

To detect the HCV-NS3 antigen, tissue speci-
mens were cut and mounted on slides coated with 3-
aminopropyltriethoxysilane (APES). Air-dried
frozen sections were placed in 1.5% hydrogen perox-
ide/methanol for 10 min. After washing in running
tap water for 5 min, 1600 mL of 0.01 M sodium cit-
rate buffer (pH 6.0) was brought to a boil in a
Prestige stainless steel pressure cooker using a hot-
plate. When the maximum temperature was reached,
sections were incubated in this solution for 1 min
and then washed in Tris-buffered saline (TBS) for 5
min. Sections were then placed in diluted normal
serum for 20 min and covered with a primary
Novocastra mouse monoclonal antibody against
HCV-NS3 (Leica Microsystems, Wetzlar, Germany).
After 5 min of washing in TBS, sections were incu-
bated in a diluted anti-mouse peroxidase-conjugated
antibody for 30 min. After washing in TBS for an
additional 5 min, sections were incubated in the
appropriate dilution of mouse peroxidase-antiperoxi-
dase (PAP) for 30 min. Sections were then washed in
TBS and incubated in diluted diaminobenzidine
tetrahydrochloride (DAB). Finally, specimens were
counterstained with hematoxylin, dehydrated, cov-
ered with a coverslip, and mounted. Specimens were
then ready for observation by a pathologist (KF Lee)
using a light microscope. The specimen processing
steps were performed according to the manufactur-
er’s instructions (Leica Microsystems). The intensity
of HCV-NS3 staining present in hepatocytes was
semiquantitatively scored as 0–3+: 0, no positively
stained cells; 1+, fewer than 33% positive cells; 2+,
34%–66% positive cells; 3+, 67%–100% positive
cells/per high-power field (Fig. 1A–C). A tissue sam-
ple that was HCV-Ab negative but HBsAg positive
was stained as a control (Fig. 1D).

**Statistical methods**

Analysis of variance (ANOVA), Chi-squared

---

**Fig. 1** Representative microscopic images of immunohistochemical staining for the hepatitis C virus (HCV)-NS3 protein in liver biopsy specimens. (A) Score 0, no positively stained cells. (B) Score 1+, < 33% positive cells (arrowhead). (C) Score 2+, 34%–66% positive cells. (D) Negative control. 400 x magnification.
tests, and Kruskal-Wallis tests were used where appropriate. Data analysis was conducted using SPSS version 17 (SPSS, Chicago, IL, U.S.A.). ANOVA was used for continuous data analysis, and the Chi-squared test was used for ordinal data.

RESULTS

Hepatocyte HCV-NS3 expression in patients with chronic hepatitis C

After excluding certain patients based on the conditions described above, 214 patients (114 males and 100 females) with complete data were enrolled in this study. Patient ages ranged 21~79 (57.65 ± 11.1) years at the time of the biopsy. In total, 113 patients were HCV genotype 1, and 97 patients were genotype 2. The clinical characteristics of all patients are summarized in Table 1.

Among the 214 liver specimens from patients positive for HCV-Ab, 202 (94%) stained positive for the HCV-NS3 protein. The intensity of HCV-NS3 staining was 0 in 13 (6%), 1+ in 181 (84%), and 2+ in 21 (10%) patients.

Hepatocyte HCV-NS3 expression in patients with chronic hepatitis B

Six patients with chronic hepatitis B who did not have chronic hepatitis C were enrolled as a control group (HBsAg [+], HCV [-]). Among these 6 patients, 2 had serum ALT levels of < 2-fold the upper limit of ALT, 2 had ALT levels of 2~5-fold the upper limit of ALT, and 2 had ALT levels > 5-fold the upper limit of ALT. Liver tissue samples from these 6 control patients were all negative for HCV-NS3 protein expression. The specificity of the IHC staining method used in this study was 100%.

Hepatocyte HCV-NS3 expression and liver histology

To determine the correlation between the HCV-NS3 staining intensity and other clinicopathological parameters, we further analyzed those parameters relative to the staining scores (Table 2).

In total, 141 (65.6%) had HAI scores of < 11, and 74 (34.4%) patients had scores of > 11. The HAI scores did not increase relative to the hepatocyte HCV-NS3 staining intensity (p = 0.861). We further evaluated the odds ratio (OR) of the degree of the HAI score based on the HCV-NS3 staining intensity.

The HAI OR was 0.72 (95% confidence interval (CI): 0.221~2.373, p = 0.592) in the HCV-NS3 staining intensity 0/1 group and 0.968 (95% CI: 0.371~2.520, p = 0.946) in the HCV-NS3 staining intensity 1/2 group. After subdividing the HAI scores into inflammation and fibrosis scores, we still observed no correlation of the staining intensity of the HCV-NS3 protein with liver inflammation or fibrosis (p = 0.700 and 0.959, respectively).

HCV-NS3 expression and serum ALT levels

Serum ALT levels in patients with 0, 1+, and 2+ HCV-NS3 staining were 134.8 ± 76.6, 163.7 ± 119.5, and 211.2 ± 190.1 IU/L, respectively. Serum ALT levels tended to increase as HCV-NS3 staining intensity increased, but this was not statistically significant (p = 0.177, ANOVA).

Table 1. Clinical Characteristics of the 214 Patients in This Study

| Age (years) | 57.65 ± 11.11 |
| Male: female (no.) | 114: 100 |
| AST (U/L) | 108.28 ± 62.62 |
| ALT (U/L) | 166.74 ± 126.53 |
| HCV-RNA (106 IU/mL) | 1.21 ± 2.48 |
| HAI score (10~22) | 9.65 ± 2.63 |
| Inflammation score (0~18) | 7.07 ± 2.34 |
| Fibrosis score (number, %) | 6.54% |
| Genotype (1: 2) (no.) | 113: 97 |
| Anti-HCV (S/CO) (mean ± S.D.) | 36.16 ± 46.82 |
| Non-SVR: SVR (no.) | 25: 86* |
| HCV-NS3 (0+: 1+: 2+) (no.) | 12: 181: 21 |

Abbreviations: AST: aspartate aminotransferase; ALT: alanine transaminase; HCV: hepatitis C virus; HAI: hepatitis activity index; S/CO: sample/cut-off ratio; SVR: sustained viral response; *: Only 111 patients had data for post-treatment HCV-RNA levels. Data are presented as the number value or mean ± standard deviation (S.D.).
Hepatocyte HCV-NS3 expression and the serum anti-HCV titer

The mean serum anti-HCV titers (sample/cutoff (S/CO) ratios) in patients with 0, 1+, and 2+ NS3 staining were 60.29, 36.69, and 26.4, respectively. Serum S/CO ratios tended to increase as the HCV-NS3 staining intensity increased, but this was not statistically significant (p = 0.139).

Hepatocyte NS3 expression and the serum HCV RNA load

Mean serum HCV RNA levels of patients with 0, 1+, and 2+ HCV-NS3 staining were 0.47 x 10^6, 0.58 x 10^6, and 0.36 x 10^6 IU/mL, respectively. Serum HCV RNA levels did not increase as the HCV-NS3 staining intensity increased (p = 0.854, ANOVA).

Hepatocyte HCV-NS3 expression and the treatment response

Among the participants in this study, 111 patients received standard treatment of pegylated interferon plus ribavirin for chronic hepatitis C and had complete virology data, and 86 (77.48%) of these patients achieved a sustained viral response. The staining intensity of the HCV-NS3 protein in liver tissues did not significantly differ between patients with a successful or failed response (p = 0.861, Chi-squared test).

**DISCUSSION**

Detection and localization of HCV in liver tissues are vital for diagnostic purposes and clinical management of patients infected with HCV, and to elucidate virological mechanisms. The fragility of HCV-RNA and the low levels of viral expression in infected tissues are substantial limitations to molecular assays for HCV characterization. HCV antigen detection in liver biopsies by IHC is an attractive option for the precise localization and quantification of viral proteins and allows direct access to histological patterns. Currently, there are few IHC methods targeting specific HCV proteins, such as NS3, NS4a, and NS5a.
NS5a, and NS5b. Among these proteins, HCV-NS3 expression in hepatocytes is known to correlate well with the presence of HCV RNA in liver tissue. Therefore, the HCV-NS3 protein should be a good indicator of the viral load in the liver. In this study, HCV-NS3 staining demonstrated a high sensitivity (94%) for detecting hepatocyte HCV.

Several studies investigated the association between the severity of liver disease and HCV proteins in liver tissues, and their conclusions were controversial. Attallah et al. found that the NS4 detection rate increased with the progression of liver disease. Galy et al. also showed that stronger E2 glycoprotein staining was related to more-advanced liver fibrosis. On the other hand, other studies did not demonstrate similar findings. Kasprak et al. found that tissue expressions of HCV-NS3 and core proteins did not correlate with the histologic grade or stage of liver tissues in patients with chronic hepatitis C. As the case number in that previous study was not large and the sensitivity of the IHC staining method was low, the role of IHC staining in HCV proteins probably could not be fully exhibited. In the present study, we used a more-sensitive method and enrolled a larger number of patients; however, our results still showed that hepatocyte HCV-NS3 expression did not correlate with the severity of liver disease. We also found that the intensity of hepatocyte HCV-NS3 expression did not correlate with serum ALT levels, anti-HCV S/CO ratios, or HCV RNA levels. These findings imply that liver damage caused by HCV might not be directly cytopathic or dose-dependent. To validate the use of hepatocyte HCV protein expression as a marker of disease severity, a further understanding of the pathogenesis of HCV-related liver injury is needed.

Recent publications showed that an IHC assessment of HCV antigens is a good tool for differentiating and managing acute liver injury after liver transplantation. However, no previous study investigated the correlation between hepatocyte HCV antigen expression and treatment outcomes. We attempted to determine if the intensity of hepatocyte HCV-NS3 expression was an outcome predictor for HCV treatment in this study, but we did not observe a positive correlation.

In summary, IHC staining of hepatocyte HCV proteins is a sensitive tool for identifying the presence of HCV in patients with chronic hepatitis. The intensity of HCV protein staining, however, did not correlate with the histological severity, serum HCV RNA load, ALT level, anti-HCV S/CO ratio, or treatment outcome. Further investigations into the pathogenesis of HCV-related liver injury are warranted.

Acknowledgements

This study was supported by Chang Gung Memorial Hospital, Chiayi, Taiwan.

REFERENCES

10. Knodell RG, Ishak KG, Black WC, Chen TS, Craig R, Kaplowitz N, Kiernan TW, Wollman J. Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active
肝組織上C型肝病毒NS3蛋白質表現與肝切片病理分級
或臨床肝炎活性不相關

廖歲宣 董水義 沈建亨 李錦輝\* 吳正雄

背 景：慢性B型肝炎的患者，其肝組織上的HbcAg已被證實與血清中的ALT高低、DNA高低及肝組織病理上受損程度有正相關。然而在慢性C型肝炎患者，少有人探討有無類似的關係。本項研究目的在於探討，C肝病毒的NS3蛋白質在肝組織上的染色強度是否和臨床上一些常見的C型肝炎檢測有相關。

方 法：本研究共收集了214位慢性C型肝炎病人的臨床資料、血清學資料和肝切片。利用針對NS3蛋白質的單株抗體來做肝組織上的NS3免疫組織化學染色，將染色強度由0至3+半定量後，分析染色強度是否和血清中的HCV-RNA、AST、ALT高低以及肝切片的HAI score有正相關。

結 果：在214位病人中，共有204位可染出，敏感度達94%。半定量的染色強度在2+的有21位（10%），在1+的181位（84%），有12位未能染出（6%），沒有病人的切片可達強度3+。經分析，染色強度和病人的年紀（p=0.302,ANOVA test），性別（p=0.130,Fisher’s exact test），ALT高低（p=0.177,ANOVA test），血清RNA量（p=0.305,ANOVA test），血清HCV抗體高低（p=0.139,Chi-square test），肝切片HAI score（p=0.861,Chi-square test），或是治療反應（sustained viral response）（p=0.861,Chi-square test）皆無相關。

結 論：因高敏感度，肝切片上的NS3蛋白質免疫組織化學染色法提供我們一項C型肝病毒的檢測法。然而，其染色強度和血清中的病毒量、肝炎活性、肝組織受損程度均無正相關，在對於慢性C型肝炎治療的反應預測方面也沒有幫助。慢性C型肝炎的致病機轉需要更多的研究，本染色法或許可以是一項不錯的工具。

(長庚醫誌2011;34(2):260-7)

關鍵詞：C型肝炎病毒NS3蛋白質，免疫組織化學染色法，HAI分數