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Intrahepatic HCV RNA Level and Genotype 1 Independently Associate with Hepatic Reticulon 3 Expression

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Abstract. *Background /Aim: Reticulon 3 (RTN3), resides predominantly in the endoplasmic reticulum and has opposite regulatory effects on Hepatitis C virus (HCV) replication through interacting with NS5A and NS4B proteins. This study aimed to unravel the actual effect of RTN3 on HCV replication. Materials and Methods: A total of 115 HCV-related hepatocellular carcinoma patients receiving hepatectomy was enrolled in this study. The hepatic HCV RNA and RTN3 protein levels in the non-cancerous liver tissues were examined for clinical analysis. Results: Of the 115 patients, 16 (11.5%) were occult HBV infection (positive for tissue HBV DNA. Univariate followed by multivariate analysis revealed that intrahepatic RTN3 levels were independently associated with higher HCV viral load (p=0.018) and HCV genotype 1 (p=0.017). Multivariate analysis revealed that HCV genotype 1, tumor size, albumin and aspartate transaminase associated with a shorter recurrence-free survival (p<0.05). Conclusion: Higher intrahepatic RTN3 levels independently correlated with higher intrahepatic HCV RNA levels and genotype 1 HCV.*

Chronic hepatitis C virus (HCV) infection is a major global health problem that can lead to severe consequences such as liver cirrhosis and hepatocellular carcinoma (HCC), and an overall increase in the liver-related morbidity and mortality (1-3). HCV, a member of the *Flaviviridae* family, has a genome that contains a positive-sense, single-stranded RNA of approximately 9.6 kb in length encoding a polyprotein of ~3,000 amino acid (4, 5). The structural (components of the mature virus) and non-structural (NS) proteins (functional elements to help viral replication) are produced through a proteolytical process (6-8). The seven NS proteins (P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) are involved in different stages of viral replication (9).

Similar to other positive-strand RNA viruses, the sites of HCV RNA replication is believed to be concentrated on ER-associated membranous compartments, although the molecular details remain to be elucidated (6). Of the NS proteins, NS4B is a 27-kDa ER membrane-associated protein, which comprises of four transmembrane (TM) domains at its central portion, two consecutive amphipathic α -helical domains (AH1 and AH2) at the N-terminal region, and two helices (H1 and H2) at the C-terminal portion (9-11). NS4B self-oligomerizes via the AH2 domain, facilitating the formation of the "membranous web", which is believed to be critical for efficient HCV viral replication (12). On the other hand, NS5A is an RNA binding hydrophilic phosphoprotein, that exists in a dimeric form without trans-membrane helices (13). It consists of three domains with distinct functions (14). NS5A also modulates the polymerase activity of NS5B, an RNA-dependent RNA polymerase (15). Consequently, NS4B and NS5A have emerged as attractive and promising drug targets for antiviral therapy.

The reticulon (RTN) family proteins reside predominantly in ER with more than 200 members. They are 200-1,200 amino acids (aa) in length (16), sharing a reticulon homology domain of 150-201 aa in carboxyl-terminal regions. Based on the sequence homology, it was classified into four families: reticulon 1, 2, 3 and 4/Nogo. In general, RTN family proteins mainly exist in nervous tissues, whereas RTN3 and RTN4B are expressed ubiquitously (9, 17). RTN primarily plays a role in promoting membrane curvature development, nuclear pore

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complex formation, vesicle maturation, DNA binding autophagy, and inflammatory-related functions (18, 19). Notably, an interaction between RTN3 and NS4B proteins has been confirmed from screening a liver cDNA library using a yeast-two hybrid system (20). It has been reported that RTN3 acts as a restriction factor for HCV replication due to its ability to compete for and bind to the AH2 domain of NS4B (9). However, another study discovered that RTN3 promotes HCV propagation by interfering with the NS5A protein (14).

RTN3 has been associated with both inhibition and promotion of HCV life cycle. This controversial role of RTN3 in the HCV life cycle and its actual function in HCV-infected patients remains elusive. In this study, we clarified this important issue regarding use of clinic liver tissues from patients with HCV-related HCC.

Patients and Methods

Ethics. The research was ethically conducted in accordance with the World Medical Association Declaration of Helsinki. All patients enrolled, signed an informed consent before submitting their surgical samples to the tissue bank of Chang Gung Medical Center for research use. This study was conducted under the approval of Institutional Review Board, Chang Gung Medical Center, Taiwan. All patients were identified by numbers and no real names were used during the study.

Patients. A total of 115 patients diagnosed as HCC with chronic HCV infection and receiving surgical resection were enrolled during July 2002 to August 2007 in Chang Gung Memorial Hospital. All patients were negative for serum Hepatitis B virus (HBV) surface antigen (HBsAg). The diagnosis of HCC was confirmed by liver biopsy, aspiration cytology and/or high alpha-fetoprotein (AFP) levels (>400 ng/ml) plus two dynamic image studies (dynamic computer tomography and angiography). Patients with anti-HCV seropositive tissues and detectable HCV RNA for more than 24 weeks were identified as infected with chronic HCV. The occult HBV infection was defined as the persistence of HBV genomes in the blood or liver of individuals with negative serum HBsAg testing (21). Clinicopathological parameters were recorded, including gender, age, HBsAg, alcohol usage, clinical cirrhosis, Edmondson's histology grade, microvascular invasion, macrovascular invasion, presence of tumor capsule, number of tumors, largest tumor size (in diameter) and AFP. The albumin, aspartate transaminase (AST), alanine transaminase (ALT), bilirubin, creatinine, and prothrombin time were determined with biochemistry and hemogram analysis.

Serum anti-HCV antibody was assayed by a third-generation enzyme immunoassay kit (AxSYM HCV Version 3.0; Abbott Laboratories). Serum HBsAg was detected by radioimmunoassay (Ausria-II, HBsAg-RIA; Abbott Laboratories).

HCV RNA quantification and genotype determination of non-cancerous liver tissues. Hepatic HCV-RNA levels were quantified following a previous study (22). Briefly, RNA was extracted from liver tissues (para-neoplastic, non-cancerous parts) using TRIzol reagents (Invitrogen, Carlsbad, CA). HCV RNA detected by COBAS TagMan HCV test (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's protocol. The final levels of hepatic HCV RNA were calculated as IU per gram with a lower detected limit of 1,500 IU/g. HCV genotype was determined using the InoLipa method (COBAS AmpliPrep/COBAS TaqMan HCV Test, Roche Diagnostics, Basel, Switzerland).

Table I. *Basic clinical data of 115 HCV-related HCC patients included in this study.*

Parameters	Value
Sample size, number of patients	115
Gender, Male/Female	78/37
Age, years, Mean±SD	62.8±11.0
Occult HBV, Positive/Negative	16/99
Cirrhosis, Yes/No	77/38
Microvascular invasion, Yes/No	38/77
Macrovascular invasion, Yes/No	14/101
Histology grade, ≤2/>2	40/75
Capsule, Yes/No	89/26
Tumor number, 1/≥1	68/47
Tumor size, cm, Mean±SD	4.4±3.0
Alcoholism, Yes/No	17/98
AFP, ng/mL, Median (range)	37.9 (<2-16057)
Albumin, g/dL, Mean±SD	3.9±.6
Bilirubin, mg/dL, Mean±SD	1.2±1.5
Prothrombin Time, sec, Mean±SD	12.2±1.3
Creatinine, mg/dL, Mean±SD	1.2±0.9
AST, U/L, Median (range)	51 (13-937)
ALT, U/L, Median (range)	59 (7-1462)
HCV-RNA, Median (range)	733.3 (0->500000.0)
Genotype, 1/non-1	67/48

Hepatic HBV DNA detection. Liver tissues were homogenized in DNA extraction buffer (10 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.2% SDS, 0.2 M NaCl and 100 μg/mL proteinase K) and incubated at 56˚C for 3 h. After addition of RNase A and incubated at room temperature for 1 h, the homogenates were extracted once with phenol/chloroform followed by chloroform extraction. The DNA was precipitated by adding NaOAc, glycogen, and alcohol. The DNA pellet was washed with 70% alcohol, dried, and dissolved in pure water. The quantitation of HBV DNA was examined by COBAS TagMan HBV test (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's protocol. The lowest detection limit of this assay was 60 IU/g (23).

Hepatic RTN3 protein extraction and Western blot analysis. Total protein was extracted from liver tissues using RIPA lysis buffer. Proteins were separated in a 10% SDS-PAGE gel and transferred to a PVDF membrane (PerkinElmer, Boston, MA). Antibodies against RTN3 used in Western blot analysis were raised against full-length human RTN3 (9). The protein-antibody complex was detected by the chemiluminescent substrate (Cell Signaling Technology, Danvers, MA). The intensities of bands were semi-quantified by Image J software. GAPDH was usedas a loading control, and was detected using anti-GAPDH antibody (6C5; Novus Biologicals, Littleton, CO).

Table II. *Comparison of clinical parameters between patients with low and high intrahepatic RTN3 levels.*

Parameters	Low RTN3	High RTN3	*p*-Value
Sample size, number of patients	60	55	
Gender, Male/Female	39/21	39/16	0.663
Age, years, Mean±SD	63.0±11.3	62.6±10.8	0.848
HBV DNA, Positive/Negative	7/53	9/46	0.647
Cirrhosis, Yes/No	39/21	38/17	0.788
Microvascular invasion, Yes/No	21/39	17/38	0.788
Macrovascular invasion, Yes/No	8/52	6/49	0.913
Histology grade, <=2/>2	19/41	21/34	0.591
Capsule, Yes/No	49/11	40/15	0.357
Tumor number, 1/≥1	38/22	30/25	0.443
Tumor size, cm, Mean±SD	4.2±2.7	4.7±3.3	0.397
Alcoholism, Yes/No	10/50	7/48	0.74
AFP, ng/mL, Median (range)	46.0 (<2-16057)	27.0 (3-4819)	0.327
Albumin, g/dL, Mean±SD	3.9±.5	3.9±.6	0.94
Bilirubin, mg/dL, Mean±SD	1.2±1.9	1.2±1.1	0.976
Prothrombin Time, sec, Mean±SD	12.0±1.0	12.4±1.5	0.119
Creatinine, mg/dL, Mean±SD	1.1±0.6	1.2±0.1	0.51
AST, U/L, Median (range)	53.5 (20-288)	48 (13-937)	0.926
ALT, U/L, Median (range)	62.5 (17-426)	56 (7-1462)	0.849
HCV-RNA, Median (range)	517.5 (0-45333.3)	2493.3 (0->500000)	0.005
Genotype, 1/non-1	25/35	42/13	<0.000

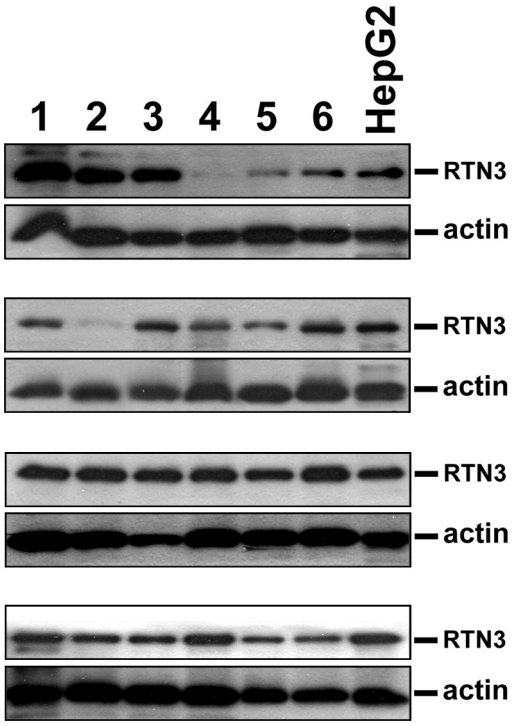

Figure 1. *Western blot analysis of RTN3 expression levels. Protein was extracted from the non-cancerous parts of liver tissues. For each batch of experiment, protein extracted from HepG2 cells were loaded in parallel (to the right). After densitometry analysis, all RTN3 levels were normalized with the corresponding actin levels. RTN3 expression levels were calculated as relative fold of RTN3 abundance in HepG2 cells. The RTN3 expression level in HepG2 cells was assigned as 1-fold. Because of the non-linear nature, patients were dichotomized using the mean value of RTN3 expression levels as a cut-off to form high and low expression groups for subsequent statistical analysis.*

Table III. *Multivariate logistic regression analysis to estimate association of the effect of two virological parameters on tissue RTN3 levels.*

Parameter	Odd Ratio	95% CI	*p*-Value
HCV genotype 1	2.846	1.202-6.734	0.017
HCV-RNA (×1000 IU/g)	1.053	1.009-1.099	0.018

Statistical analysis. Dichotomous data were reported as numbers and percentages and compared using chi-square test or Fisher's exact tests, where appropriate. Parametric data were reported as means±SD for continuous variables with normal distribution and as median (range) for continuous variables with a non-normal distribution. For comparisons between groups, the two-sample Student's *t*-test was used for continuous variables with normal distribution, and the Mann-Whitney *U*-test was used for continuous variables with a non-normal distribution. The parametric data were dichotomized into two groups, with the mean values used as cut-offs. To evaluate the relationship between the intrahepatic RTN3 levels and clinicopathological parameters as well as virological parameters, univariate and multivariate logistic regression analysis were performed. Recurrence-free survival was calculated from the date of treatment to the date of HCC recurrence or last follow-up. Overall survival was calculated from the date of treatment to the date the patient deceased or had the last follow-up. Univariate and multivariate Cox proportional hazard models were used to estimate recurrence-free survival and overall survival for clinicopathological parameters, virological parameters, and intrahepatic RTN3 levels. Those variables that were statistically significant in the univariate analysis at the level of $p<0.05$ were included in the multivariate analysis. All tests were two-tailed, and a *p*-value of less than 0.05 was considered statistically significant. All statistical analyses were performed using the Statistical package for the social sciences (SPSS) for Windows Version 18.0 (SPSS, Chicago, IL).

Results

Basic characteristics of patients. A total of 115 HCV-related HCC patients receiving hepatectomy were enrolled in the study. Their baseline demographic and clinical features are summarized in Table I. The mean age was 62.8 (range=41-78) ±11.0 years and 78 (67.8%) patients were male. The mean AST and ALT levels were mildly higher than the upper

Table IV. *Factors associated with recurrence-free survival and overall survival.*

	Recurrence-free survival					Overall survival		
		Univariate		Multivariate		Univariate	Multivariate	
Parameter	Patient No	*p*-Value	HR	95%CI	*p*-Value	*p*-Value	HR	95%CI
Age, years								
≤62	48							
>62	67	0.822				0.970		
Gender								
Female	37							
Male	78	0.738				0.173		
HBV-DNA								
Negative	99							
Positive	16	0.031	1.610	0.766-3.383	0.209	0.162		
HCV-RNA								
≤733.3	58							
>733.3	57	0.843				0.761		
Alcoholism								
No	98							
Yes	17	0.292				0.459		
Ascites								
No	107							
Yes	8	0.324				0.409		
Cirrhosis								
No	38							
Yes	77	0.825				0.570		
Macrovascular invasion								
No	101							
Yes	14	0.491				0.799		
Microvascular invasion								
No	77							
Yes	38	0.634				0.759		
Histology grade								
≤2	40							
>2	75	0.894				0.720		
Capsule								
No	26							
Yes	89	0.530				0.672		
Tumor size, cm								
≤4.4	71							
>4.4	44	0.050	2.019	1.128-3.612	0.018	0.140		
Tumor No								
1	68							
>1	47	0.303				0.643		
Biochemistry								
Bilirubin, mg/dl								
≤1.2	85							
>1.2	30	0.375				0.976		
Albumin, g/dl								
≤3.9	53							
>3.9	62	0.007	0.536	0.307-0.936	0.028	0.767		
Creatinine, mg/dl								
≤1.2	95							
>1.2	20	0.227				0.321		
AST, U/l								
≤51	58							
>51	57	0.004	1.286	1.112-1.488	0.001	0.876		
ALT, U/l								
≤59	59							
>59	56	0.052				0.998		

Table IV. *Continued*

Table IV. *Continued*

	Recurrence-free survival					Overall survival		
		Univariate		Multivariate		Univariate	Multivariate	
Parameter	Patient No	*p*-Value	HR	95%CI	*p*-Value	*p*-Value	HR	95%CI
Prothrombin time, sec								
≤12.2	75							
>12.2	40	0.399				0.979		
AFP, ng/ml								
≤38	58							
>38	57	0.523				0.018	11.88	1.533-92.607
Genotype								
Non-1	48							
1	67	0.003	2.776	1.551-4.978	0.001	0.383		
RTN3 level								
Low	60							
High	55	0.526				0.763		

limit of normal (51 U/L and 59 U/L, respectively). Sixteen (11.5%) patients had occult HBV infection with detectable intrahepatic HBV DNA in the non-cancerous liver tissue. The pathological parameters of most tissues revealed, liver cirrhosis, HCV genotype 1, Edmondson's histologic grade >2, tumor capsule, and tumor numbers ≥1.

Higher RTN3 expression is independently correlated with higher HCV viral loads and genotype 1. To clarify the role of RTN3 in HCV replication under physiological conditions, total protein extracted from liver tissues was examined for RTN3 expression by western blot (Figure 1). After normalization with actin, the RTN3 expression in HepG2 cells was defined as 1 to estimate the relative fold difference of RTN3 expression levels in liver tissues. The RTN3 levels were dichotomized into low- and high-expressing groups by using mean values as a cut-off. The RNA extraction from the same specimens was used for HCV RNA analysis. The correlation between the expression level of intrahepatic RTN3 and the clinical and virological factors was analyzed. As shown in Table II, the positive correlation between expression levels of RTN3 and HCV-RNA was observed. The patients with higher intrahepatic RTN3 levels had higher HCV-RNA levels (2493.3 IU/g *vs*. 517.5 IU/g; $p=0.005$). The high expression of intrahepatic RTN3 was also found to be significantly correlated with genotype 1 (42/55 (76.4%) *versus* 25/60 (41.7%); $p<0.001$). As for other clinical parameters including age, sex, occult HBV infection, liver cirrhosis, alcoholism, Edmondson's histological grade, microvascular invasion, macrovascular invasion, presence of tumor capsule, tumor numbers, largest tumor size, AFP, albumin, AST, ALT, bilirubin, creatinine, and prothrombin time, no statistically significant correlation was found with intrahepatic RTN3 expression levels.

Multivariate logistic regression analysis showed that higher HCV viral load (OR=1.053; 95%CI=1.009-1.099; $p=0.018$) and HCV genotype 1 (OR=2.846; 95%CI=1.202-6.734; $p=0.017$) independently correlated with high RTN3 (Table III).

Clinicopathological and virological factors associated with postoperative survival in HCV-related HCC. The cox proportional hazard model was used to examine the relationship between the recurrence-free survival and clinicopathological factors, virological factors, and intrahepatic RTN3 levels. As shown in Table IV, HCV genotype 1, combined occult HBV infection, tumor size >4.4 cm, albumin ≤3.9 g/dl, and ALT >51 U/l were associated with a shorter recurrence-free survival using Univariate analysis. After adjusting other confounders, multivariate analysis revealed that HCV genotype 1 ($p=0.001$), tumor size >4.4 cm ($p=0.018$), albumin ≤3.9 g/dL ($p=0.028$), and ALT >51 U/L ($p=0.001$) were significantly associated with a shorter recurrence-free survival. Similarly, the association between overall survival and clinicopathological factors, virological factors, and intrahepatic RTN3 levels was also analysed using the Cox proportional hazard model (Table IV). Only an AFP level greater than 38 ng/ml associated with a shorter overall survival in Univariate analysis.

Discussion

Owing to the discovery of HCV in 1989, the technique of HCV RNA detection can now be used to accurately identify patients that are infected with HCV. It has been found that

most HCV-infected patients (80-85%) fail to clear the virus following acute infection, and thus subsequently progress to chronic stages. Chronic HCV infection may lead to various complications including cirrhosis, portal hypertension, hepatic decompensation, and the development of HCC. Up to 350,000 patients die per year due to HCV infection (24). Therefore, how to eradicate chronic HCV infection remains presently a critical issue. As such, identification of viral and host factors involved in HCV replication is a key prerequisite for the discovery of new antiviral drug targets in the future. RTN3 plays a crucial role in HCV replication (9, 14, 20), however, the conflicting regulatory effects of RTN3 in HCV replication were found from studying different HCV viral proteins. Two HCV non-structural proteins (NS4B and NS5A) which play important roles in HCV replication physically interact with RTN3. Studies have shown that, RTN3 can compete for and bind to the AH2 domain of NS4B restricting HCV replication by abolishing AH2-mediated NS4B self-interaction (9). On the contrary, RTN3 promotes HCV propagation by interfering NS5A (14). To date, no studies have clarified the interaction between these two, NS4B and NS5A, non-structural proteins and RTN3 at the same time and its effect on HCV replication. In this study we tried to shed light on this puzzling issue through direct assessment of intrahepatic RTN3 levels and investigation of its correlation with various clinicopathological and virological factors.

According to our clinical analysis, the higher intrahepatic RTN3 levels were significantly associated with higher intrahepatic HCV viral loads in liver tissue. This finding is consistent with the study which enhanced HCV propagation through the effects of NS5A-RTN3 interaction, suggesting that increased viral propagation may overcome the interference of viral replication (NS4B-RTN3 interaction effect) in order to maintain a higher HCV RNA level.

Apart from proving the clinical correlation between RTN3 and HCV replication, we discovered that higher intrahepatic RTN3 levels also associated with HCV genotype 1 (42/67 *vs.* 13/48, $p<0.001$). In addition, HCV genotype 1 was shown to be an independent factor for predicting high intrahepatic RTN3 levels ($p=0.017$). To our knowledge, this finding has never been reported before. The molecular mechanism of this novel finding remains unclear. Nevertheless, since HCV viral load and genotype 1 are important predictors of response to antiviral treatment (24), we speculate the intrahepatic RTN3 levels could potentially predict outcome of antiviral therapies.

Taiwan is an area hyperendemic for HBV infection. The majority of patients are infected by the age of 3 through perinatal transmission (25, 26). However, HBV-infected persons rarely progress into a chronic status if the infection occurs after the age of 3 (21). Therefore, the cases with occult HBV infection included in this study were probably chronic HBV carriers superinfected by HCV, resulting in loss of HBV surface antigen (27). In this view, HBV served as an initiator and HCV served as a promoter of hepatocarcinogenesis. In the present study, the presence of occult HBV infection ($p=0.031$) was associated with unfavorable recurrence-free survival in univariate Cox analysis. However, the expression levels of intrahepatic HCV RNA was unable to predict the postoperative survival of patients. These findings are in accordance with a previous study (22). Other survival predictors included tumor size, albumin, ALT, and AFP, suggesting the prognostic role of tumor burden, liver functional reserve, and continuous hepatic inflammation (28). In our analysis, the RTN3 levels did not play a role in predicting postoperative outcome.

The major limitation of the current study is that the number of liver tissues obtained from HCV-associated HCCs remained relatively small because most HCC patients (>60%) in Taiwan were HBsAg-positive.

Conclusion

This study demonstrated that higher intrahepatic RTN3 levels were independently associated with higher intrahepatic HCV viral loads and genotype 1 in HCV-related HCC.

Conflicts of Interest

All Authors declare no conflict of interest.

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References

1 EASL Clinical Practice Guidelines: management of hepatitis C virus infection. J Hepatol *60*: 392-420, 2014.

2 El-Serag HB: Epidemiology of viral hepatitis and hepatocellular carcinoma. Gastroenterology *142*: 1264-1273, 2012.

3 Perz JF, Armstrong GL, Farrington LA, Hutin YJ and Bell BP:The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. J Hepatol *45*: 529-538, 2006.

4 Choo QL, Richman KH, Han JH, Berger K, Lee C, Dong C, Gallegos C, Coit D, Medina-Selby R, Barr PJ, Weiner AJ, Bradley DW, Kuo G and Houghton M: Genetic organization and diversity of the hepatitis C virus. Proc Natl Acad Sci USA *88*: 2451-2455, 1991.

5 Hnatyszyn HJ: Chronic hepatitis C and genotyping: the clinical significance of determining HCV genotypes. Antivir Ther *10*: 1-11, 2005.

6 Elazar M, Cheong KH, Liu P, Greenberg HB, Rice CM and Glenn JS: Amphipathic helix-dependent localization of NS5A mediates hepatitis C virus RNA replication. J Virol *77*: 6055-6061, 2003.

7 Kuiken C and Simmonds P: Nomenclature and numbering of the hepatitis C virus. Methods Mol Biol *510*: 33-53, 2009.
8 Bartenschlager R, Penin F, Lohmann V and Andre P: Assembly of infectious hepatitis C virus particles. Trends Microbiol *19*: 95-103, 2011.
9 Wu MJ, Ke PY, Hsu JT, Yeh CT and Horng JT: Reticulon 3 interacts with NS4B of the hepatitis C virus and negatively regulates viral replication by disrupting NS4B self-interaction. Cell Microbiol *16*: 1603-1618, 2014.
10 Einav S, Elazar M, Danieli T and Glenn JS: A nucleotide binding motif in hepatitis C virus (HCV) NS4B mediates HCV RNA replication. J Virol *78*: 11288-11295, 2004.
11 Elazar M, Liu P, Rice CM and Glenn JS: An N-terminal amphipathic helix in hepatitis C virus (HCV) NS4B mediates membrane association, correct localization of replication complex proteins, and HCV RNA replication. J Virol *78*: 11393-11400, 2004.
12 Gouttenoire J, Roingeard P, Penin F and Moradpour D: Amphipathic alpha-helix AH2 is a major determinant for the oligomerization of hepatitis C virus nonstructural protein 4B. J Virol *84*: 12529-12537, 2010.
13 Huang Y, Staschke K, De Francesco R and Tan SL: Phosphorylation of hepatitis C virus NS5A nonstructural protein: a new paradigm for phosphorylation-dependent viral RNA replication? Virology *364*: 1-9, 2007.
14 Tripathi LP, Kambara H, Chen YA, Nishimura Y, Moriishi K, Okamoto T, Morita E, Abe T, Mori T, Matsuura Y and Mizuguchi K: Understanding the biological context of NS5A-host interactions in HCV infection: a network-based approach. J Proteome Res *12*: 2537-2551, 2013.
15 Macdonald A and Harris M: Hepatitis C virus NS5A: tales of a promiscuous protein. J Gen Virol *85*: 2485-2502, 2004.
16 Oertle T, Klinger M, Stuermer CA and Schwab ME: A reticular rhapsody: phylogenic evolution and nomenclature of the RTN/Nogo gene family. FASEB J *17*: 1238-1247, 2003.
17 Chiurchiu V, Maccarrone M and Orlacchio A: The role of reticulons in neurodegenerative diseases. Neuromolecular Med *16*: 3-15, 2014.
18 Voeltz GK, Prinz WA, Shibata Y, Rist JM and Rapoport TA: A class of membrane proteins shaping the tubular endoplasmic reticulum. Cell *124*: 573-586, 2006.
19 Yang YS and Strittmatter SM: The reticulons: a family of proteins with diverse functions. Genome Biol *8*: 234, 2007.
20 Diaz A and Ahlquist P: Role of host reticulon proteins in rearranging membranes for positive-strand RNA virus replication. Curr Opin Microbiol *15*: 519-524, 2012.
21 Hsu HY, Chang MH, Chen DS, Lee CY and Sung JL: Baseline seroepidemiology of hepatitis B virus infection in children in Taipei, 1984: a study just before mass hepatitis B vaccination program in Taiwan. J Med Virol *18*: 301-307, 1986.
22 Chang ML, Lin YJ, Chang CJ, Yeh C, Chen TC, Yeh TS, Lee WC and Yeh CT: Occult and Overt HBV Co-Infections Independently Predict Postoperative Prognosis in HCV-Associated Hepatocellular Carcinoma. PLoS One *8*: e64891, 2013.
23 Lin CL, Chien RN, Hu CC, Lai MW and Yeh CT: Identification of hepatitis B virus rtS117F substitution as a compensatory mutation for rtM204I during lamivudine therapy. J Antimicrob Chemother *67*: 39-48, 2012.
24 Webster DP, Klenerman P and Dusheiko GM: Hepatitis C. Lancet *385*: 1124-1135, 2015.
25 Lin CL, Chien RN, Yeh C, Hsu CW, Chang ML, Chen YC and Yeh CT: Significant renoprotective effect of telbivudine during preemptive antiviral therapy in advanced liver cancer patients receiving cisplatin-based chemotherapy: a case-control study. Scand J Gastroenterol *49*: 1456-1464, 2014.
26 Chen CJ, Wang LY and Yu MW: Epidemiology of hepatitis B virus infection in the Asia-Pacific region. J Gastroenterol Hepatol *15 Suppl*: E3-6, 2000.
27 Kaklamani E, Trichopoulos D, Tzonou A, Zavitsanos X, Koumantaki Y, Hatzakis A, Hsieh CC and Hatziyannis S: Hepatitis B and C viruses and their interaction in the origin of hepatocellular carcinoma. JAMA *265*: 1974-1976, 1991.
28 Fujiwara Y, Shiba H, Furukawa K, Iida T, Haruki K, Gocho T, Wakiyama S, Hirohara S, Ishida Y, Misawa T, Ohashi T and Yanaga K: Glasgow prognostic score is related to blood transfusion requirements and post-operative complications in hepatic resection for hepatocellular carcinoma. Anticancer Res *30*: 5129-5136, 2010.

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