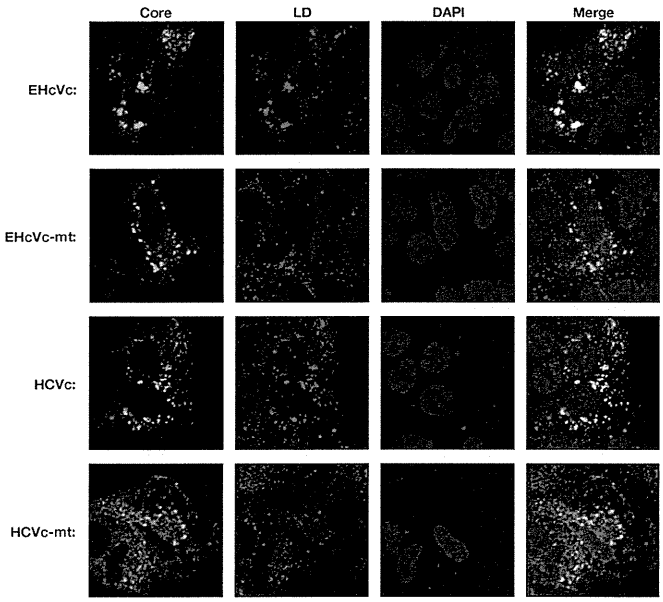

FIG 7 Intracellular localization of hepacivirus core proteins. HCVc, HCVc-mt, EHcVc, or EHcVc-mt was expressed in the Huh7OK1 cell line. The resulting cells were stained with Bodipy 558/568 (red) and then fixed with 4% paraformaldehyde at 24 h posttransfection, permeabilized, and subjected to indirect immunofluorescence staining. Each core protein was detected using mouse anti-FLAG antibodies and then Alexa 488-conjugated anti-mouse IgG (green). Cell nuclei were stained with DAPI after fixation (blue).

many (5, 7–9). Although the infection route of EHcV remains unknown, horses that were previously imported to Japan may be highly infected with EHcV. The serological prevalence in the present study appeared to be lower than that reported previously (8). A specific signal of the viral protein may be selected by Western blotting, used herein, rather than by the luciferase immunoprecipitation system, as reported previously (8), since the serum of each horse reacted to different proteins irrespective of the EHcV core protein (Fig. 2). The predicted full sequence of the EHcV strain amplified from serum sample 3 had high homology to those of the previously reported strains (Table 2). The polyproteins of previous strains had approximately 95% amino acid homology to one another irrespective of the area in which the horses originated, suggesting that these strains may belong to the same virological species. The parents of horse number 3 were born in Japan, while its grandparents were imported from the United States and Canada. Unfortunately, the sera of the parents and grandparents were not obtained in the present study. The EHcV strains obtained from Japanese-born horses may have originated from the United States or Canada. Another possibility is that one species of EHcV may have recently been distributed worldwide.

The primary and secondary structures of both UTRs are conserved among HCV strains and are essential for replication and translation. Four major stem-loop (SL) motifs have been detected in the 5′ UTR of the HCV genome, three SL structures of which are known to be required for IRES activity (46). Domain IIId plays a crucial role in anchoring of the 40S ribosome for IRES activity (47). Domain IIIb and the four-way helical junction of domains IIIa, IIIb, and IIIc bind eukaryotic initiation factor 3 (eIF3) and form a ternary complex, thereby forming the 48S preinitiation complex on HCV RNA (48). Moreover, domain II is known to be required to enhance eIF5-mediated GTP hydrolysis and the release of eIF2 from the 48S complex (48). These equivalent motifs were observed in the predicted secondary structures of the 5′ UTR of the reported EHcV strains (49), as well as in strain JPN3/JAPAN/2013 in the present study (Fig. 4C). A recent study demonstrated that the EHcV 5′ UTR exhibited IRES-dependent translation activity (50); however, further studies are needed to fully understand the IRES activity of the EHcV 5′UTR.

SL motifs embedded in the NS5B-coding region and UTRs of the HCV genome are known to be associated with viral replication. Several studies found that the mutational disruption of the complement sequence between 5BSL3.2 and 3′SL2 inhibited HCV RNA replication (33, 51). Additionally, the apical loop of domain IIId in the HCV 5′ UTR was shown to interact with the bulge of 5BSL3.2, supporting IRES-dependent translation and viral RNA replication (34–36). The RNA secondary structures of the 3′ UTR in the EHcV genome remained unknown due to limited information on its nucleotide sequence. 3′ RACE using poly(U) polymerase was employed in the present study because the ordinary 3′-RACE reaction using poly(A) polymerase was stopped at the (A)-rich region of the EHcV 3′ UTR. The nucleotide sequence of the EHcV 3′ UTR was determined, and its RNA secondary structure was then predicted (Fig. 4B and C). The results of the present

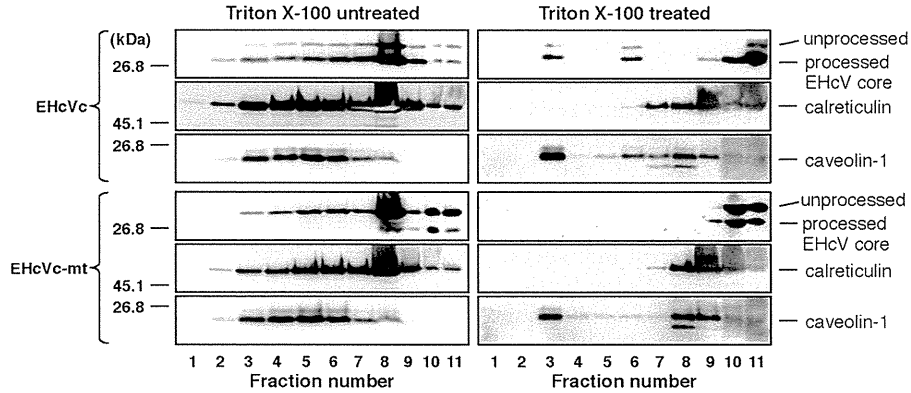

FIG 8 The EHcV core protein partially migrated to the DRM fraction after SPP-dependent processing. 293FT cells expressing either EHcVc or EHcVc-mt were homogenized with or without 1% Triton X-100 and then subjected to a flotation assay. Proteins in each fraction were concentrated with cold acetone and then subjected to Western blotting using the anti-FLAG, anti-calreticulin, or anti-caveolin-1 antibody.

study revealed that the 3′ UTR of the EHcV genome consists of the (A)-rich sequence and relatively shorter 3′-X-tail sequence. The three SL structures of the EHcV 3′ UTR were similar to those of the HCV 3′ UTR but were markedly different from the 3′ UTRs of GBV-B and rodent hepaciviruses. Drexler et al. described the structural characteristics of the 5′ and 3′ UTRs in rodent hepacivirus as well as phylogenetic information, liver tropism, and the pathogenicity of the virus (5). The SL motifs embedded in the 3′ X-tails of rodent hepacivirus and GBV-B varied. The structures of the 3′ UTRs appeared to correspond to the phylogenetic relationship of the hepaciviruses (5). Figure 4C shows that there were two stem-loop structures within the NS5B-coding region of EHcV corresponding to 5BSL3.2 and 5BSL3.3 of HCV RNA. Complementary regions were observed between the 5BSL3.2-like domain and 3′SL2, as well as between the 5BSL3.2-like domain and domain IIId of the EHcV genome (Fig. 4B and C). The kissing-loop and long-range RNA-RNA interactions may be structurally conserved between EHcV and HCV. Functional analyses of the *cis*-acting elements of the EHcV genome will contribute to the establishment of an EHcV infection system.

The mature HCV core protein was previously shown to be generated from the viral precursor polyprotein by signal peptidase followed by SPP-dependent processing of the transmembrane region (52). The core proteins of HCV and GBV-B are known to be cleaved by SPP (12, 37). The transmembrane regions of both the HCV and EHcV core proteins were found to be structurally conserved, based on their amino acid sequences and hydrophobicity plots (Fig. 5A and B and Fig. 6B). The replacement of Ile^{190} and Phe^{191} with Ala and Leu, respectively, in the EHcV core protein abrogated the intramembrane processing of the EHcV core protein (Fig. 6C). The loss-of-function mutant of SPP inhibited intramembrane processing of the EHcV core protein (Fig. 6D). Furthermore, the loss-of-function mutant of SPP specifically interacted with an uncleaved form of the EHcV core protein (Fig. 6E). These results indicate that the transmembrane region of the EHcV core protein may have been cleaved by SPP. The mature HCV core protein is known to be translocated into LDs and partially on lipid raft-like membranes. Previous studies reported that the HCV core protein on the LDs may be recruited near the replication complex in the membranous web, which consists of cholesterol- and sphingolipid-rich lipid components (43–45). Viral assembly was shown to occur on the ER membrane close to LDs and the membranous web (14). In addition to the HCV core protein, the nonstructural proteins and viral RNA of HCV were detected in the DRM fractions. The HCV RNA polymerase NS5B was previously reported to interact with sphingomyelin (53). Furthermore, a serine palmitoyltransferase inhibitor suppressed HCV replication by disrupting the replication complex (53, 54). These findings indicate that the DRM is provided as a scaffold for the formation of the HCV replication complex (45, 54). In the present study, we showed that the mature EHcV core protein was localized mainly on LDs and partially on the DRM (Fig. 7 and 8). A mutational analysis of the EHcV core protein indicated that SPP-dependent cleavage may be required for the localization of the EHcV core protein on LDs and the DRM in a manner similar to that for the HCV core protein. In addition, the assembly mechanism of EHcV may be similar to that of HCV.

In conclusion, the results of the present study show that EHcV shares common features with the HCV genomic structure and the biological properties of the capsid protein. *In vivo* and *ex vivo* infection systems for EHcV have not yet been successfully established. SCID mice carrying chimeric human livers are currently employed as a small animal model for *in vivo* infection with HCV (55) but are not suitable for studies on immunity and pathogenicity due to an immunodeficiency. Chimpanzees are not yet available for *in vivo* HCV research. Further studies on the mechanisms underlying EHcV infection will contribute to the development of an *in vivo* surrogate model system for studying HCV immunity and pathogenicity.

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Hepatology Research

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Original Article

Liver stiffness measurement for risk assessment of hepatocellular carcinoma

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Aim: Liver fibrosis is a risk factor for hepatocellular carcinoma (HCC), but at what fibrotic stage the risk for HCC is increased has been poorly investigated quantitatively. This study aimed to determine the appropriate cut-off value of liver stiffness for HCC concurrence by FibroScan, and its clinical significance in hepatitis B virus (HBV), hepatitis C virus (HCV) and non-B, non-C (NBNC) liver disease.

Methods: Subjects comprised 1002 cases (246 with HCC and 756 without HCC) with chronic liver disease (HBV, 104; HCV, 722; and NBNC, 176).

Results: Liver stiffness was significantly greater in all groups with HCC, and the determined cut-off value for HCC concurrence was more than 12.0 kPa in those with HCV, more than 8.5 kPa in those with HBV and more than 12.0 kPa in those with NBNC. Liver stiffness of more than 12.0 kPa was an independent risk factor for new HCC development in HCV. For HCV, risk factors for HCC concurrence were old age, male sex, low albumin, low platelets and liver stiffness, while for HBV they were old age, low platelets and liver stiffness, and for NBNC they were old age, elevated α-fetoprotein and liver stiffness.

Conclusion: Liver stiffness cut-off values and their association with HCC concurrence were different depending on the etiology. In HCV, liver stiffness of more than 12.0 kPa was an independent risk factor for new HCC development. Collectively, determining the fibrotic cut-off values for HCC concurrence would be important in evaluating HCC risks.

Key words: FibroScan, hepatocellular carcinoma, liver fibrosis

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is the fifth most common cancer in the world and the third most common cause of cancer deaths.[1] HCC, accounting for 90% of primary liver cancer, is a global clinical issue.[2] For improvement in the prognosis of HCC, curative therapy following early detection is important. To this end, it is critical to identify high-risk groups for HCC and perform appropriate surveillance in the clinical practice of chronic liver disease. It has been postulated that hepatitis virus infection, old age, male sex, alanine aminotransferase (ALT) elevation, liver fibrosis, and low albumin (Alb), low platelets (Plt) and α-fetoprotein (AFP) elevation are risk factors for HCC; however, liver fibrosis is the most important risk factor irrespective of its etiology.[3–6]

To date, liver fibrosis has been evaluated by liver biopsy, but it is associated with several problems such as invasiveness, sampling errors, semiquantitation and diagnostic differences among pathologists. With the development of FibroScan (Echosens, Paris, France) using transient elastography, it has become possible to quantitate liver elasticity non-invasively.[7] The diagnostic accuracy of FibroScan for liver fibrosis has been recognized widely for various chronic liver diseases with the exception of some liver conditions such as congestion,

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severe inflammation or cholestasis in which liver fibrosis might be overestimated with FibroScan.[8–12] The risk for HCC is evaluable based on liver stiffness measured by FibroScan in cases with hepatitis B virus (HBV) and hepatitis C virus (HCV).[12–19] Nevertheless, in most reports the risk for HCC was only indirectly evaluated based on the value for liver cirrhosis as measured by FibroScan. Liver stiffness related to HCC has not been directly evaluated. Furthermore, the utility of FibroScan in evaluation of the risk for HCC has not been elucidated in non-B, non-C (NBNC) liver disease.

In this study, liver stiffness in patients with chronic liver disease was quantitatively measured and liver stiffness related to HCC occurrence was elucidated separately in cases with HCV, HBV and NBNC liver disease for investigations of its clinical utility.

METHODS

Patients

THE SUBJECTS COMPRISED 1002 patients with chronic liver disease whose liver stiffness was measured by FibroScan consecutively at the University of Yamanashi Hospital between January 2010 and December 2012. Informed consent had been obtained for measurement of liver stiffness before the modality was approved by the national insurance in October 2011. The HCV group (722 cases including 66 sustained virological response [SVR] cases), HBV group (104 cases) and NBNC group (176 cases) were defined as HCV antibody positive, hepatitis B surface antigen (HBsAg) positive, and HBsAg negative and HCV antibody negative cases, respectively. Both HBsAg and HCV antibody positive cases ($n = 3$) and HIV co-infection cases (co-infection with HBV, $n = 1$) were excluded. HCC cases included those with a history of HCC. Among the 1002 cases with chronic liver disease, 246 had HCC and 756 were without HCC. Of those without HCC, 470 hepatitis C cases were followed up by abdominal ultrasonography, contrast computed tomography (CT) or ethoxybenzyl (EOB) contrast magnetic resonance imaging (MRI) every 3–6 months. HCC was diagnosed by contrast ultrasonography, contrast enhancement in the arterial phase and poor enhancement at the equilibrium phase in contrast CT (including CT arteriography and computed tomographic arterial portography) and contrast MRI, and histology by liver tumor biopsy. According to the Declaration of Helsinki, this study was performed after approval was obtained by the ethical committee of the Faculty of Medicine, University of Yamanashi.

Measurement of liver stiffness

FibroScan502 (Echosens) was used for measurement with the M-probe and L-probe. Patients were placed in a supine position with the right hand at the most abducted position for right intercostal scanning. When at least 10 effective measurements were obtained with effective measurement at 60% or higher and interquartile range at less than 30%, such measurements were defined as effective and the median was employed as the result of the measurement.[20]

Analytical methods

In each group of liver diseases (HCV, HBV and NBNC), liver stiffness was compared between patients with and without HCC. Then, the cut-off value of liver stiffness for diagnosis of HCC was determined for later analysis in each group. Patients' backgrounds, laboratory data and liver stiffness in the HCV, HBV and NBNC groups were subjected to univariate, multivariate and subgroup analyses on the relationship with HCC. The 470 HCV patients without HCC at enrollment were followed up with the day of measurement of liver stiffness designated as day 0. Factors related to the development of HCC were examined by univariate and multivariate analyses using values for liver stiffness and blood test results at enrollment.

Statistical analysis

Category data were analyzed by the χ^2-test and Fisher's exact test, while numerical data were examined by Mann–Whitney *U*-test. The cut-off value was set to yield the largest Youden index by receiver–operator curve (ROC) analysis. Multiple logistic analysis was performed for multivariate analysis on factors related to HCC concurrence. The Cox regression hazard model was employed for multivariate analysis of factors related to HCC development. Yearly development of HCC was expressed as per person•year. Cumulative incidence of HCC development was calculated by the Kaplan–Meier curve. *P*-values less than 0.05 were considered significant.

RESULTS

Baseline characteristics

CLINICAL BACKGROUND FACTORS of 1002 patients were compared between patients with and without HCC according to group (Table 1). There were 722 cases in the HCV group, 104 in the HBV group and 176 in the NBNC group. For all groups there was a significant association with older age, low Alb and Plt,

Table 1 Baseline characteristics of patients with and without HCC

Factors	HCV patients ($n = 722$)			HBV patients ($n = 104$)			NBNC patients ($n = 176$)		
	HCC(+) ($n = 167$)	HCC(−) ($n = 555$)	*P*	HCC(+) ($n = 29$)	HCC(−) ($n = 75$)	*P*	HCC(+) ($n = 50$)	HCC(−) ($n = 126$)	*P*
Age (years)	72 (42–89)	61 (20–89)	<0.01	62 (49–76)	52 (19–73)	<0.01	70 (53–88)	63 (19–88)	<0.01
Sex (male/female)	111/56	288/266	<0.01	23/6	47/28	0.11	33/17	69/58	0.16
Alb (g/dL)	3.6 (1.8–5.1)	4.3 (2.1–5.3)	<0.01	4.4 (2.0–5.0)	4.5 (3.5–5.2)	0.04	3.8 (1.9–4.7)	4.1 (2.4–5.5)	<0.01
T-Bil (mg/dL)	0.8 (0.3–4.7)	0.7 (0.2–26.9)	<0.01	0.7 (0.3–1.2)	0.7 (0.2–1.6)	0.45	0.7 (0.1–1.5)	0.7 (0.1–2.3)	0.90
AST (U/L)	48 (13–340)	32 (8–262)	<0.01	28 (16–95)	25 (14–178)	0.06	43 (17–146)	32 (10–291)	0.03
ALT (U/L)	43 (4–557)	32 (2–334)	<0.01	25 (10–134)	21 (9–375)	0.13	29 (10–80)	29 (6–517)	0.99
γ-GT (U/L)	36 (11–918)	28 (9–354)	<0.01	56 (13–267)	21 (8–222)	<0.01	74 (15–628)	55 (7–743)	0.14
Plt (10^9/L)	94 (25–299)	157 (40–343)	<0.01	118 (21–207)	172.5 (58–300)	<0.01	117 (14–264)	168 (30–387)	<0.01
AFP (ng/mL)	12.9 (1.3–54 923)	3.6 (0.8–839)	<0.01	3.8 (1.3–22 421)	2.7 (1.1–70.9)	<0.01	5.8 (1.3–5194)	3.2 (0.8–25.3)	<0.01
Stiffness (kPa)	21.3 (3.9–75.0)	7.8 (3.0–72.0)	<0.01	9.2 (4.7–75.0)	5.6 (2.8–32.4)	<0.01	15.6 (3.3–75.0)	7.4 (2.8–66.4)	<0.01
Hx of IFN Tx (yes/no)	38/129	153/402	0.21	–	–	–	–	–	–
SVR/non-SVR	10/34	56/97	0.09	–	–	–	–	–	–
Tx of NA	–	–	–	16/13	34/41	0.37	–	–	–
HBV-DNA >4 log copies/mL	–	–	–	4/25	16/59	0.38	–	–	–

Values are expressed as the mean (range).

–, Not applicable; AFP, α-fetoprotein; Alb, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBV patients, HBs antigen positive patients; HCC, hepatocellular carcinoma; HCV patients, HCV antibody positive patients; Hx, history; IFN, interferon; NA, nucleoside analog; NBNC patients, HBs antigen negative and HCV antibody negative patients; Plt, platelet count; stiffness, liver stiffness; SVR, sustained virological response; T-Bil, total bilirubin; Tx, Treatment; γ-GT, γ-glutamyl transpeptidase.

and elevated AFP among those with HCC. The proportion of males was significantly higher among the HCC cases in the HCV group. Stiffness of the liver was significantly greater among the HCC cases in all groups.

Determining cut-off values related to HCC concurrence in each disease group

The cut-off value most related to HCC concurrence was determined by the ROC analysis in each disease group. It was set at more than 12.0 kPa (>12.0 kPa vs ≤12.0 kPa; odds ratio [OR], 14.7; $P < 0.001$) in the HCV group, at more than 8.5 kPa (>8.5 kPa vs ≤8.5 kPa; OR, 8.28; $P < 0.001$) in the HBV group and at more than 12.0 kPa (>12.0 kPa vs ≤12.0 kPa; OR, 4.67; $P < 0.001$) in the NBNC group (Fig. 1).

HCC concurrence-related factors

Hepatocellular carcinoma concurrence-related factors in the HCV group were examined. Univariate analysis revealed that age, sex, Alb, total bilirubin, aspartate aminotransferase (AST), γ-glutamyltransferase (γ-GT), Plt, AFP and liver stiffness of more than 12.0 kPa were significant factors (Table 2). With the significant factors extracted by univariate analysis, multivariate analysis was performed, and age, sex, Alb, Plt and liver stiffness of more than 12.0 kPa were independent factors (Table 3). Liver stiffness of more than 12.0 kPa was significant with an OR of 4.53 ($P < 0.001$).

Hepatitis C virus patients were categorized into two groups according to liver stiffness of 12.0 kPa or less, and more than 12.0 kPa, and HCC concurrence-related factors were examined in each group. Multivariate analysis extracted age, sex, Alb and AFP in the group with liver stiffness of 12.0 kPa or less as independent factors, and age, Alb and Plt in the group with liver stiffness of more than 12.0 kPa (Table 3).

In the HBV group, HCC concurrence-related factors were examined. Univariate analysis revealed that age, Alb, γ-GT, Plt, AFP and liver stiffness of more than 8.5 kPa were significant factors (Table 2), and multivariate analysis extracted age as an independent factor (OR, 1.12 [range, 1.04–1.21], $P < 0.004$) while low Plt tended to be associated with a high risk for HCC occurrence (OR, 0.99 [range, 0.98–1.00], $P = 0.08$) (data not shown). Subgroup analysis showed that liver stiffness of more than 8.5 kPa was a significant factor for HCC concurrence irrespective of age of more than 60 years or 60 years or less, and Plt less than 150×10^9/L or 150×10^9/L or more (Fig. 2).

Also examined were HCC concurrence-related factors in the NBNC group. Univariate analysis revealed that Alb, Plt, AFP and liver stiffness of more than 12.0 kPa

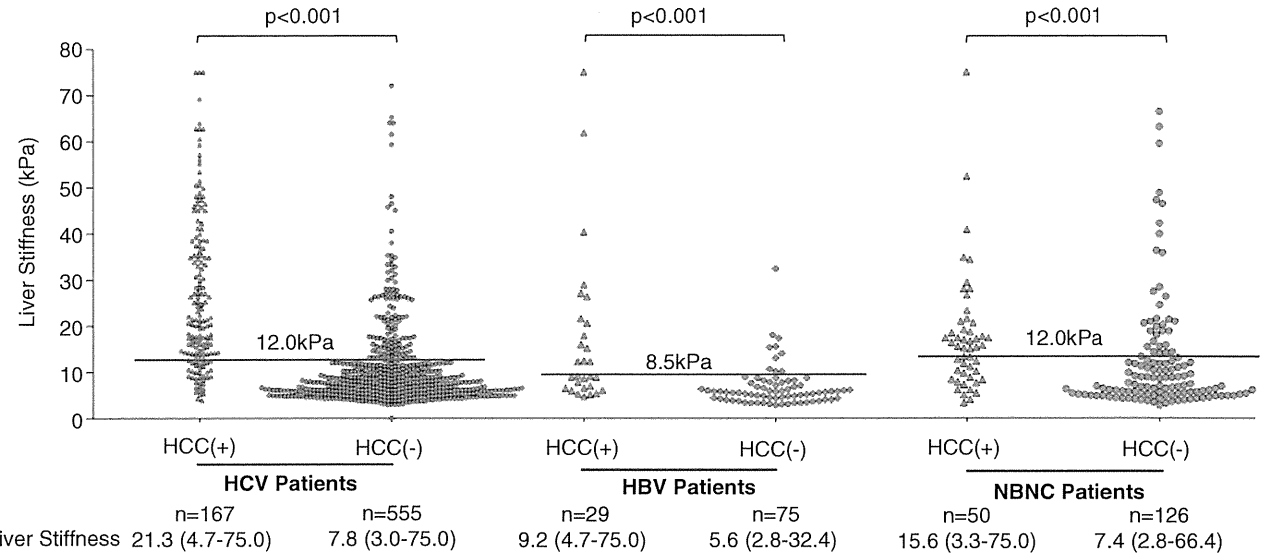


Figure 1 Distribution of liver stiffness categorized by the presence of hepatocellular carcinoma (HCC). Distribution of liver stiffness is shown in cases with liver disease of different etiologies with and without HCC. The cut-off value for liver stiffness was calculated so that sensitivity plus specificity would be the largest. A horizontal line indicating the cut-off value was drawn separately in each etiology group with an insertion of the value. Liver stiffness is shown as the median (range). Liver stiffness scores were significantly higher in cases with HCC concurrence. HBV, hepatitis B virus; HCV, hepatitis C virus; NBNC, non-B, non-C.

Table 2 Factors related to HCC: univariate analysis

Factors	HCV patients ($n = 722$)			HBV patients ($n = 104$)			NBNC patients ($n = 176$)		
	OR	95% CI	*P*	OR	95% CI	*P*	OR	95% CI	*P*
Age (years)	1.13	1.11–1.16	<0.001	1.09	1.04–1.14	<0.001	1.07	1.04–1.12	<0.001
Sex (male)	1.84	1.28–2.64	0.001	2.28	0.83–6.29	0.110	1.66	0.84–3.27	0.147
Alb (g/dL)	0.07	0.04–0.11	<0.001	0.20	0.07–0.59	0.003	0.33	0.17–0.63	<0.001
T-Bil (mg/dL)	1.53	1.09–2.14	0.014	1.20	0.24–6.02	0.826	0.80	0.32–2.03	0.639
AST (U/L)	1.01	1.01–1.02	<0.001	1.01	0.99–1.02	0.431	1.00	0.99–1.01	0.554
ALT (U/L)	1.00	0.99–1.01	0.103	0.99	0.99–1.01	0.868	0.99	0.98–1.00	0.281
γ-GT (U/L)	1.00	1.00–1.01	0.005	1.02	1.01–1.03	0.003	1.00	0.99–1.00	0.392
Plt (10^9/L)	0.98	0.97–0.98	<0.001	0.98	0.97–0.99	0.001	0.99	0.98–0.99	<0.001
AFP (ng/mL)	1.01	1.01–1.02	<0.001	1.04	1.00–1.08	0.033	1.14	1.04–1.26	0.007
Stiffness > cut-off value*	14.3	9.27–22.1	<0.001	7.13	2.76–18.4	<0.001	4.67	2.32–9.40	<0.001
Hx of IFN Tx (yes/no)	0.77	0.51–1.15	0.208	–	–	–	–	–	–
SVR patients	0.56	0.28–1.13	0.108	–	–	–	–	–	–
NA Tx	–	–	–	1.48	0.63–3.51	0.369	–	–	–
HBV DNA >4 log copies/mL	–	–	–	0.21	0.05–1.01	0.051	–	–	–

*The cut-off value is 8.5 kPa in HBV patients, and 12.0 kPa in HCV and NBNC patients.
–, Not applicable; AFP, α-fetoprotein; Alb, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBV patients, HBs antigen positive patients; HCC, hepatocellular carcinoma; HCV patients, HCV antibody positive patients; Hx, history; IFN, interferon; NA, nucleoside analog; NBNC patients, HBs antigen negative and HCV antibody negative patients; Plt, platelet count; stiffness, liver stiffness; SVR, sustained virological response; T-Bil, total bilirubin; Tx, Treatment; γ-GT, γ-glutamyl transpeptidase.

were significant factors (Table 2), and multivariate analysis extracted age and AFP as independent factors (data not shown). In the subgroup aged more than 65 years and AFP of less than 10 ng/mL, liver stiffness of more than 12.0 kPa was a significant HCC concurrence-related factor (Fig. 2).

Risk of HCC development in HCV infection

In the HCV group, the risk of HCC development was evaluated in 470 patients without HCC initially who were followed up. In contrast, evaluation of the risk of development of HCC was not possible in HBV or NBNC cases because no patient in those groups without HCC initially subsequently developed HCC during this limited observation period. These 470 HCV cases were categorized into those with liver stiffness of more than 12.0 kPa and 12.0 kPa or less based on the cut-off value determined at the analysis of HCC concurrence, and Kaplan–Meier curves for HCC occurrence were constructed. Five patients developed HCC over a median

Table 3 Factors related to HCC in HCV patients: multivariate analysis

Factors	All ($n = 722$)			≤12 kPa ($n = 460$)			>12 kPa ($n = 262$)		
	OR	95% CI	*P*	OR	95% CI	*P*	OR	95% CI	*P*
Age (years)	1.13	1.10–1.17	<0.001*	1.12	1.07–1.19	<0.001*	1.12	1.07–1.16	<0.001*
Sex (male)	3.55	1.98–6.39	<0.001*	43.4	4.88–387	<0.001*			
Alb (g/dL)	0.27	0.14–0.46	<0.001*	0.19	0.06–0.63	0.007*	0.29	0.14–0.61	0.001*
T-Bil (mg/dL)	1.21	0.66–2.22	0.526				1.02	0.52–2.02	0.946
AST (U/L)	1.00	0.99–1.00	0.419						
ALT (IU/L)							0.99	0.99–1.00	0.541
γ-GT (U/L)	1.00	0.99–1.01	0.285						
Plt (10^9/L)	0.99	0.98–0.99	0.008*	0.99	0.98–1.00	0.113	0.99	0.98–0.99	0.036*
AFP (ng/mL)	1.00	0.99–1.01	0.138	1.10	1.01–1.19	0.028*	1.00	0.99–1.01	0.159
Stiffness >12.0 kPa	4.53	2.36–8.69	<0.001*	–	–	–	–	–	–

*Statistically significant.
–, Not applicable; AFP, α-fetoprotein; Alb, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CI, confidence interval; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; NA, nucleoside analog; OR, odds ratio; Plt, platelet count; stiffness, liver stiffness; SVR, sustained virological response; T-Bil, total bilirubin; γ-GT, γ-glutamyl transpeptidase.

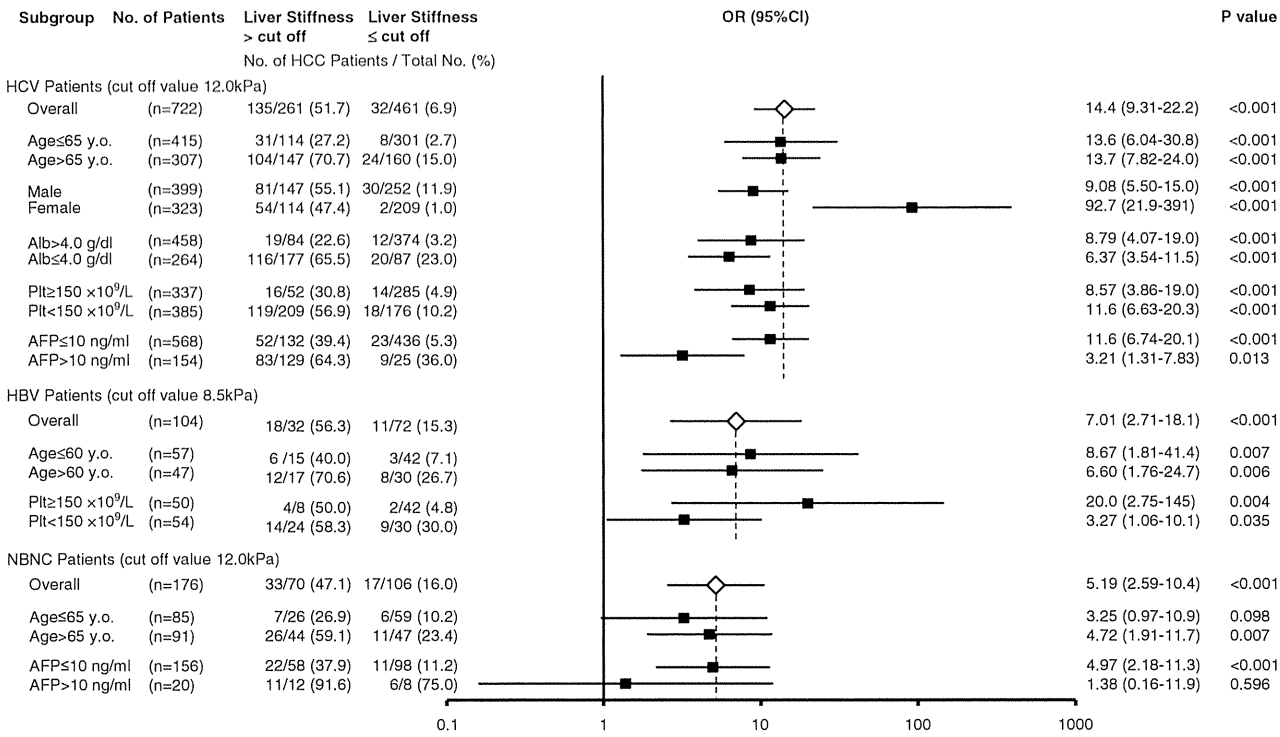


Figure 2 Odds ratio (OR) for the presence of hepatocellular carcinoma (HCC) in specified subgroups associated with liver stiffness over the cut-off value. The OR (95% confidence interval [CI]) for HCC and a *P*-value are shown for each subgroup of hepatitis C virus (HCV) patients with liver stiffness >12.0 kPa, hepatitis B virus (HBV) patients with liver stiffness >8.5 kPa and non-B, non-C (NBNC) liver disease patients with liver stiffness >12.0 kPa. Liver stiffness >12.0 kPa was a HCC concurrence-related factor in all subgroups of HCV patients. In particular, the association was stronger in females than in males. In HBV patients, liver stiffness >8.5 kPa was associated with HCC concurrence irrespective of age >60 years or ≤60 years and platelets (Plt) $\geq 150 \times 10^9$/L or $< 150 \times 10^9$/L. In NBNC patients, liver stiffness >12.0 kPa was associated with HCC concurrence in the subcategory of age >65 years and α-fetoprotein (AFP) ≤10 ng/mL.

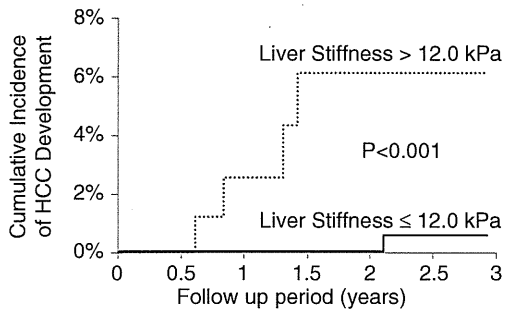
follow-up period of 691 days. The incidence of HCC development was significantly higher among cases with liver stiffness of more than 12.0 kPa than among those with liver stiffness of 12.0 kPa or less ($P < 0.001$, by log–rank test) (Fig. 3).

Factors related to HCC development were examined, and univariate analysis extracted elevated AST, elevated AFP and liver stiffness of more than 12.0 kPa as significant factors, and multivariate analysis revealed that liver stiffness of more than 12.0 kPa was an independent factor. A history of interferon treatment and a SVR were not independent risk factors (Table 4). Cumulative incidence of HCC development was 2.5% in 1 year and 6.1% in 2 years (2.63% per person•year) in patients with liver stiffness of more than 12 kPa. In those with liver stiffness of 12.0 kPa or less, it was 0% in 1 year and 0% in 2 years (0.15% per person•year).

DISCUSSION

WE FOUND THAT stiffness of the liver was significantly greater in those with HCC in the HCV, HBV and NBNC groups than among cases without HCC. In the HCV group, liver stiffness of more than 12.0 kPa was the most appropriate cut-off value for HCC concurrence producing the highest OR and the stiffness significantly correlated with HCC development. Likewise, liver stiffness of more than 8.5 kPa and more than 12.0 kPa were the most appropriate cut-off values associated with HCC concurrence in the HBV group and the NBNC group, respectively.

FibroScan has been widely used as a non-invasive measurement system for liver fibrosis. The most appropriate cut-off value for diagnosis of liver cirrhosis was 11.8–15.9 kPa with sensitivity ranging 79–87% and



No. at risk

Liver Stiffness ≤ 12.0 kPa	366	326	278	248	175	122	50
Liver Stiffness > 12.0 kPa	104	85	69	52	36	19	10

Figure 3 Cumulative incidence of hepatocellular carcinoma (HCC) development in hepatitis C virus patients. Cumulative incidence of HCC development in cases with liver stiffness >12 kPa and ≤12 kPa is shown. Four and one case developed HCC among cases with liver stiffness >12 kPa and ≤12 kPa, respectively. Liver stiffness >12 kPa was associated with a significantly higher risk of HCC development than liver stiffness ≤12 kPa ($P < 0.001$). No case with liver stiffness ≤12 kPa developed HCC for at least 2 years.

specificity 81–95% in the HCV cases, 11.7 kPa with a sensitivity of 84.6% and specificity of 81.5% in the HBV cases,[17,21–23] and 10.3–17.5 kPa with sensitivity ranging 92–100% and specificity 88–97% in non-alcoholic fatty liver disease cases.[8,11,24] On the other hand, the value for liver stiffness most significantly related to HCC concurrence not to liver cirrhosis in each disease group remains elusive.[16,18,25]

The present analysis revealed that the cut-off value most closely associated with HCC concurrence was 12.0 kPa in the HCV group. Masuzaki *et al.* reported that HCC concurrence was more frequent in the presence of a firmer liver, but presented no appropriate cut-off value.[25] In contrast, Akima *et al.* and Kuo *et al.* reported that 12.5 kPa and 12.0 kPa were, respectively, the most appropriate cut-off values for HCC concurrence. However, their studies included heterogeneous etiologies and the cut-off level was not examined separately according to each etiology.[13,16] On the other hand, these cut-off values were almost comparable with the cut-off of 12.0 kPa in the present study because most cases in these studies were positive for HCV. The cut-off level for liver stiffness at 12.0 kPa, which was most closely associated with HCC concurrence in the present study, was almost comparable to the minimum cut-off level of liver stiffness for diagnosis of liver cirrhosis. In HCV positive cases, HCC concurrence was more frequent in cases with a histological semiquantitative diagnosis of fibrosis at F4 (liver cirrhosis) by liver biopsy.[6,26,27] These clinical observations were consistent with the quantitative results of the present study.

In the HCV group, liver stiffness of more than 12.0 kPa was associated with HCC concurrence independently of other factors associated with HCC concurrence, such as age, sex, Alb and Plt (Table 3). It has been reported that male sex and old age were risk factors for HCC independent of liver fibrosis.[6,28–30] Although it is presumed that low Alb and Plt are indirectly implicated in the advancement to liver cirrhosis, liver stiffness was independent of those factors and may reflect the risk for HCC directly related to fibrosis. Subgroup analysis (Fig. 2) revealed that liver stiffness of more than 12.0 kPa was more closely associated with HCC concurrence in females than in males. It was elucidated that HCC development was more closely associated with advancement of liver fibrosis in females and that measurement of liver stiffness in females was more useful than in males.

Although it is rare, some HCV positive cases develop HCC before clinical advancement to liver cirrhosis, and the clinical characteristics of such cases have been poorly investigated. To investigate HCC concurrence-related factors, we categorized HCV positive cases into two groups according to liver stiffness of more than 12.0 kPa and 12.0 kPa or less (Table 3). In those with mild liver fibrosis with liver stiffness of 12.0 kPa or less, old age, male sex, low Alb and elevated AFP were HCC concurrence-related factors. It was suggested that the risk of developing HCC was increased even in cases with mild liver fibrosis as long as those factors were present. Recently, it was reported that metabolic factors such as diabetes and non-alcoholic steatohepatitis are associated with HCC development independently of liver fibrosis.[31–33] It is necessary to further investigate how metabolic factors influence HCC development in patients with mild liver fibrosis and low values for measurements of liver stiffness.

Furthermore, in the HCV group, 470 cases without HCC were followed up (median, 691 days), and liver stiffness of more than 12.0 kPa was the only independent factor for HCC development (hazard ratio, 12.3; 95% confidence interval, 1.27–132) (Table 4). Curves for cumulative incidence of HCC development revealed that HCC development rates were significantly different between cases with liver stiffness of more than 12.0 kPa and 12.0 kPa or less ($P < 0.001$; log-rank test) and that HCC developed beginning 6 months after measurements in cases with liver stiffness of more than 12.0 kPa, whereas no HCC developed for at least 2 years in cases

Table 4 Factors related to HCC development in HCV patients

Factors	Patients who developed HCC $n = 5$	Patients who did not develop HCC $n = 465$	Univariate			Multivariate		
			HR	95% CI	*P*	HR	95% CI	*P*
Age (years)	60 (51–72)	61 (20–88)	1.01	0.93–1.10	0.837			
Sex (male)	4 (80.0%)	245 (52.7%)	4.49	0.50–40.3	0.180			
Alb (g/dL)	4.6 (3.4–4.8)	4.3 (2.1–5.3)	1.56	0.16–15.7	0.705			
T-Bil (mg/dL)	1.2 (0.5–2.4)	0.6 (0.2–26.9)	1.10	0.86–1.40	0.442			
AST (U/L)	84 (19–131)	32 (8–262)	1.02	1.00–1.03	0.013*	1.01	0.99–1.02	0.358
ALT (U/L)	49 (13–163)	31 (2–334)	1.01	0.99–1.02	0.179			
γ-GT (U/L)	51 (12–130)	28 (9–354)	1.01	0.99–1.02	0.223			
Plt (10^9/L)	98 (82–173)	156 (43–343)	0.98	0.97–1.00	0.128			
AFP (ng/mL)	6.2 (2.1–272.8)	3.5 (0.8–839)	1.00	1.00–1.01	0.025*	1.00	0.99–1.01	0.271
History of IFN	3 (60.0%)	256 (55.1%)	0.62	0.10–3.87	0.609			
SVR patients	1 (20.0%)	124 (26.7%)	0.38	0.04–3.45	0.388			
Stiffness >12.0 kPa	4 (80.0%)	103 (22.2%)	18.9	2.10–171	<0.001*	12.9	1.27–132	0.031*
Follow-up period (days)	477 (223–963)	691 (23–1069)	–	–	–	–	–	–

*Statistically significant.

Values are expressed as the mean (range) or *n* (%).

–, Not applicable; AFP, α-fetoprotein; Alb, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CI, confidence interval; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HR, hazards ratio; IFN, interferon; NA, nucleoside analog; Plt, platelet count; stiffness, liver stiffness; SVR, sustained virological response; T-Bil, total bilirubin; γ-GT, γ-glutamyl transpeptidase.

with liver stiffness 12.0 kPa or less (Fig. 3). According to the HCC surveillance guidelines, an imaging examination every 6 months is recommended in cases with chronic hepatitis C and once in 3–4 months in cases with liver cirrhosis C.[34] In cases with liver stiffness of more than 12.0 kPa, the guidelines can be considered reasonable. In addition, in cases with liver stiffness of 12.0 kPa or less, it was suggested that the surveillance interval may be prolonged, although further accumulation of such cases was necessary.

In the HBV group, the cut-off value at 8.5 kPa most closely correlated with HCC concurrence (OR, 8.28), and both the cut-off value and OR were lower than those in the HCV group, which indicated that there was a weaker association between fibrosis and HCC in the HBV group than in the HCV group. In the HBV group, it was reported that liver stiffness at 8.0 kPa, a cut-off value lower than that in the HCV group, or higher increased the incidence of HCC development.[15] Subgroup analysis (Fig. 2) revealed that liver stiffness of more than 8.5 kPa was a significant factor irrespective of age and Plt. Unfortunately, we could not analyze the HCC developmental risk in cases with HBV because no case without concurrent HCC initially developed HCC during this limited observation period.

To the best of our knowledge, no report has demonstrated the association between liver stiffness and HCC concurrence in cases with NBNC liver disease, but when liver stiffness at 12.0 kPa was set as the cut-off value, liver stiffness most closely correlated with HCC concurrence and the cut-off value was almost comparable to that in the HCV group. This result demonstrates that fibrosis also plays an important role in HCC development in NBNC though its contribution is weaker than in HCV. Subgroup analysis revealed that HCC concurrence was more frequent in the group with liver stiffness of more than 12.0 kPa among the elderly aged more than 65 years old and cases with low AFP levels as reported previously,[32] demonstrating that the HCC risk was more greatly dependent on fibrosis in the elderly, while it was high irrespective of fibrosis in cases with elevated AFP in the NBNC group. As for etiologies in the NBNC group, most cases were clinically suspected to have fatty liver-associated diseases. Though information on steatosis-related factors was available only from limited cases in this study, high hemoglobin A1c (HbA1c) value (defined as >6.5) was frequent in NBNC cases (25%) compared to HCV (11%) or HBV cases (17%), and this difference reached statistical significance between HCV and NBNC (data not shown). In addition, high HbA1c value and heavy alcohol intake of more than 70 g/day were more significantly identified in HCC cases compared to non-HCC cases in the NBNC group (data not shown). These observations suggested that fatty liver-associated diseases may be one of the main etiologies in the NBNC group. On the other hand, as with the HBV cases, we could not analyze the HCC developmental risk in cases with NBNC because no case developed HCC during this limited observation period.

In conclusion, evaluation of liver fibrosis based on liver stiffness was useful, in particular, in HCV and NBNC liver disease, because HCC development via advancement of liver fibrosis is a major pathway. Accurate evaluation of liver fibrosis would be important to screen the high risk group for HCC development and analyze causal factors for HCC development other than fibrosis.

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DEEP SEQUENCING ANALYSIS OF VARIANTS RESISTANT TO THE NS5A INHIBITOR DACLATASVIR IN PATIENTS WITH GENOTYPE 1B HEPATITIS C VIRUS INFECTION.

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Short title: Deep sequencing for daclatasvir-resistant HCV.

Abbreviations:

HCV: hepatitis C virus, IFN: interferon, PEG: pegylated, RBV: ribavirin, SVR: sustained virological response, TPV: telaprevir, BPV: boceprevir, DAA: direct antiviral

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agent, ISDR: interferon sensitivity-determining region, IRRDR: interferon-ribavirin resistance determining region, NS3: non-structural protein 3, NS5A: non-structural protein 5A, NS5B: non-structural protein 5B, SNP: single nucleotide polymorphism, IL28B: interleukin 28B.

FOOTNOTES

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ABSTRACT

Background & Aims: Daclatasvir, an NS5A replication complex inhibitor, is a potent and promising direct antiviral agent (DAA) for hepatitis C virus (HCV), being most effective in genotype 1b infection. Although it is known that genotype 1b viruses with Y93H and/or L31M/V/F mutations have strong resistance to daclatasvir, it is not known whether there are some clinical background conditions that favor the occurrence of HCVs carrying those NS5A mutations

Methods: In this study, we carried out deep sequencing analysis of stored sera to determine the presence and significance of daclatasvir-resistant mutants in 110 genotype 1b HCV-infected patients with no previous daclatasvir treatment.

Results: Deep sequencing analysis revealed that the NS5A L31M/V/F and Y93H mutations were present in 13/110 (11.8%) and 34/110 (30.9%) patients, respectively, and significantly more frequently than in the control plasmid. Simultaneous L31M/V/F and Y93H mutations were detected in 4/110 patients (3.6%). When the clinical relevance of NS5A resistance was investigated, Y93H was significantly correlated with the IL28B major (TT) genotype of the host ($p = 0.042$).

Conclusions: Y93H was detected frequently by deep sequencing in daclatasvir treatment-naïve patients. Importantly, it seems that the IL28B status of the patients might influence the presence of Y93H mutations, resulting in different treatment responses to daclatasvir.

Key words: HCV, deep sequencing, NS5A inhibitor, resistance

INTRODUCTION

Recently, treatment of hepatitis C virus (HCV) infection has advanced markedly. Specifically, the advent of telaprevir (TPV) and boceprevir (BPV), first-generation protease inhibitors, dramatically increased the sustained virological response (SVR) rate to as high as 60% to 80% by combination with pegylated (PEG)-interferon (IFN)/ribavirin (RBV) therapy [1]. However, high SVR rates following combination therapy have not been seen in null-responders to previous PEG-IFN/RBV combination therapy [2]. Under these circumstances, development of more effective drug therapies with less serious adverse effects is anticipated.

Daclatasvir (BMS-790052), a nonstructural (NS) 5A replication complex inhibitor, is a potent and promising direct antiviral agent (DAA) for HCV. Daclatasvir has anti-HCV activity with broad genotypic coverage, but is most effective for genotype-1b viruses [3]. Moreover, among all NS5A inhibitors, daclatasvir is most advanced in its development for clinical use [4, 5]. Drug-resistant mutations have been identified for daclatasvir, and resistance is acquired by Y93H, L31M/V/F or P32L substitutions in NS5A in genotype 1b HCV. In particular, simultaneous substitutions of Y93H and L31M/V/F produce more robust resistance [6, 7].

In Japan, a clinical phase II trial of 24-week combination therapy of two oral agents, the NS5A inhibitor daclatasvir and NS3 protease inhibitor asunaprevir (BMS-650032), was carried out in 43 patients with genotype 1b HCV infection. The therapy achieved an SVR rate of 90.5% in patients with a null-response to PEG-IFN/RBV combination therapy and of 63.6% in patients considered ineligible or intolerant to IFN-based therapy [8, 9]. The result was that the SVR rate was markedly high, in particular, in patients with a null-response to PEG-IFN/RBV combination

therapy, giving hope to these difficult-to-treat patients. The study also revealed that the presence of Y93H prior to treatment was significantly associated with non-SVR to the regimen of the two oral agents [8-11]. On the other hand, it remains unknown whether differences in clinical backgrounds, including previous history of IFN therapy and its response, are associated with the presence of Y93H in daclatasvir-treatment naïve genotype 1b patients

In this study, we carried out deep sequencing analysis using a second generation sequencer to determine the presence of daclatasvir-resistant viruses in genotype 1b HCV patients. By deep sequencing, viral mutants associated with DAA resistance and present as minor populations could be detected [12-14]. Because daclatasvir is considered to be a key DAA for therapy for HCV in the near-future, we tried to clarify the possible clinical significance of HCV resistance mutations, such as Y93H, in the treatment response and their possible association with other viral and host factors.

PATIENTS AND METHODS

Patients

The subjects were 110 randomly-selected, daclatasvir treatment-naïve patients who were infected with genotype 1b HCV and followed-up at the Yamanashi University Hospital. The 110 patients included 59 naïve patients, 30 relapser patients (defined as patients with reappearance of HCV RNA after the completion of previous PEG-IFN/RBV combination therapy carried out between 2005 and 2011) and 21 null responder patients (defined as patients without a 2 log drop of HCV RNA at week 12 compared to that at week 0 during previous PEG-IFN/RBV combination therapy carried out between 2005 and 2011). These three groups of patients with distinctly different treatment responses to previous therapy (naïve, relapse, and null) were included in this study to clarify whether the rate of NS5A mutations varies among different backgrounds of the treatment response. None of the 51 patients who had failed to eradicate the virus during PEG-IFN/RBV combination therapy had received antiviral therapy thereafter. In the 110 patients, daclatasvir resistance mutations were analyzed by deep sequencing of sera collected and stored at the most recent visit to the hospital.

All patients studied fulfilled following criteria: (1) Negative for hepatitis B surface antigen. (2) No other forms of hepatitis, such as primary biliary cirrhosis, autoimmune liver disease, or alcoholic liver disease. (3) Free of co-infection with human immunodeficiency virus. (4) Signed consent was obtained for the study protocol that had been approved by Human Ethics Review Committee of Yamanashi University Hospital. The clinical backgrounds of the 110 patients are shown in Table 1.

Direct sequencing