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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 inde-

pendent 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  $\alpha$ -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

**H**EPATOCELLULAR CARCINOMA (HCC) is the fifth most common cancer in the world and the third most common cause of cancer deaths.<sup>1</sup> HCC, accounting for 90% of primary liver cancer, is a global clinical issue.<sup>2</sup> 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  $\alpha$ -fetoprotein (AFP) elevation are risk factors for HCC; however, liver fibrosis is the most important risk factor irrespective of its etiology.<sup>3-6</sup>

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.<sup>7</sup> 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.<sup>8-12</sup> 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).<sup>12-19</sup> 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.<sup>20</sup>

### 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  $\chi^2$ -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 <sup>9</sup> /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  $\leq 12.0$  kPa; odds ratio [OR], 14.7;  $P < 0.001$ ) in the HCV group, at more than 8.5 kPa ( $>8.5$  kPa vs  $\leq 8.5$  kPa; OR, 8.28;  $P < 0.001$ ) in the HBV group and at more than 12.0 kPa ( $>12.0$  kPa vs  $\leq 12.0$  kPa; OR, 4.67;  $P < 0.001$ ) in the NBNC group (Fig. 1).

### HCC concurrence-related factors

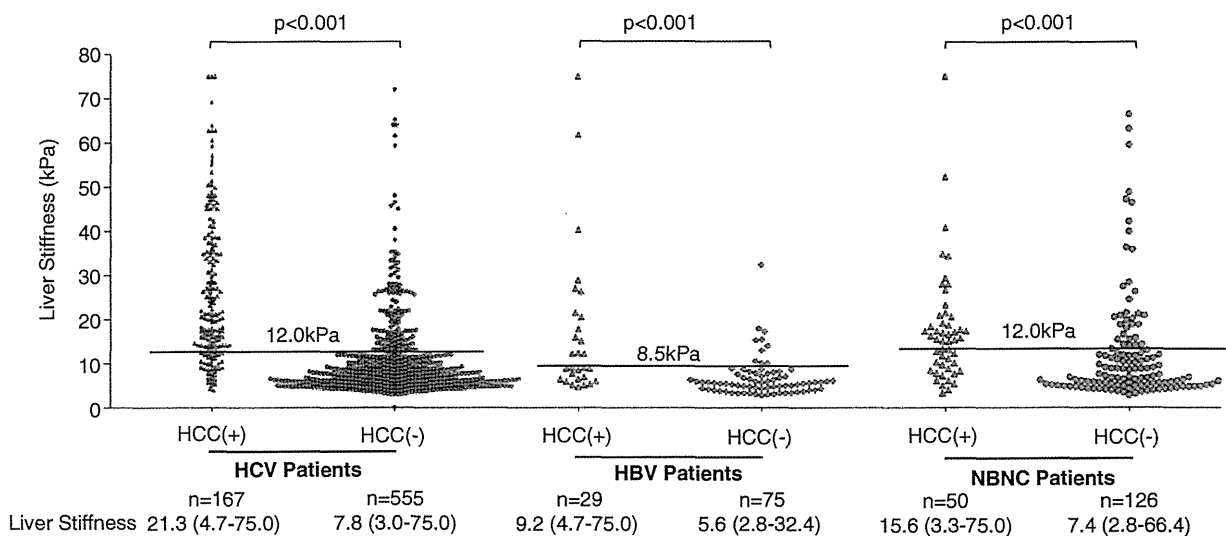
Hepatocellular carcinoma concurrence-related factors in the HCV group were examined. Univariate analysis revealed that age, sex, Alb, total bilirubin, aspartate aminotransferase (AST),  $\gamma$ -glutamyltransferase ( $\gamma$ -GT), Plt, AFP and liver stiffness of more than 12.0 kPa were significant 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,  $\gamma$ -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  $150 \times 10^9/L$  or  $150 \times 10^9/L$  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 <sup>9</sup> /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

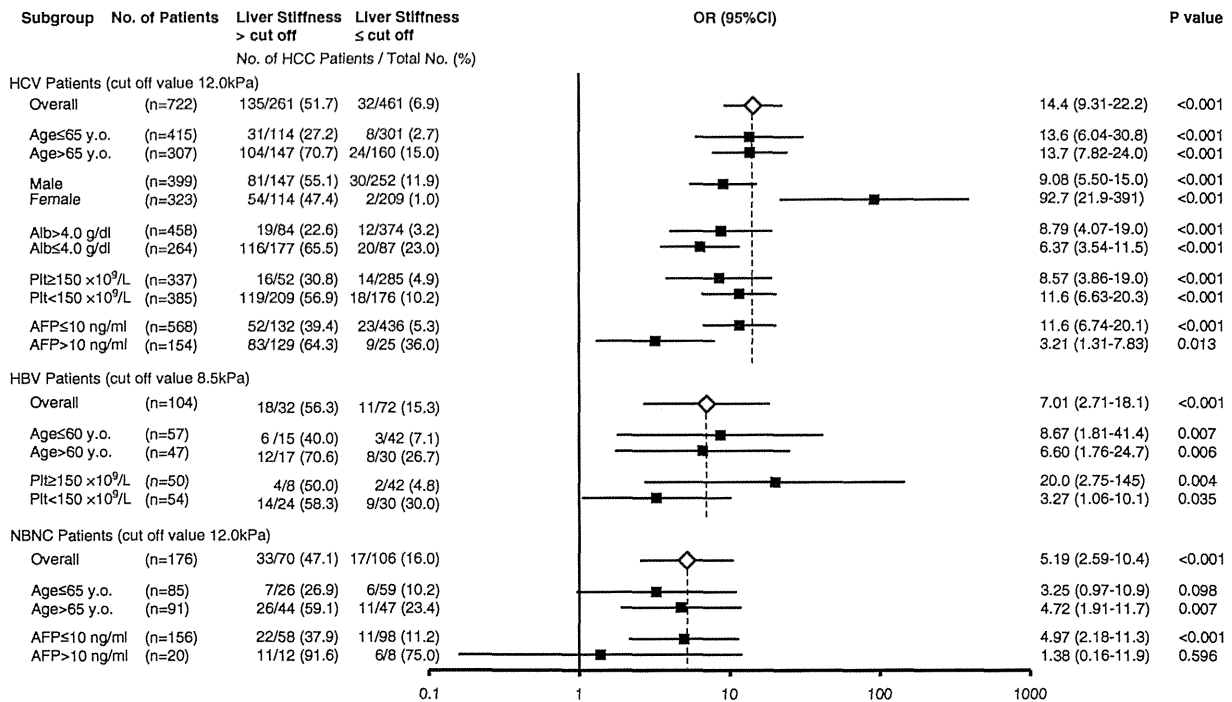
were followed up. In contrast, evaluation of the risk of development of HCC was not possible in HBV or NBNC cases because no patient in those groups without HCC initially subsequently developed HCC during this limited observation period. These 470 HCV cases were categorized into those with liver stiffness 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 <sup>9</sup> /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) ≥150 × 10<sup>9</sup>/L or <150 × 10<sup>9</sup>/L. In NBNC patients, liver stiffness >12.0 kPa was associated with HCC concurrence in the subcategory of age >65 years and  $\alpha$ -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).

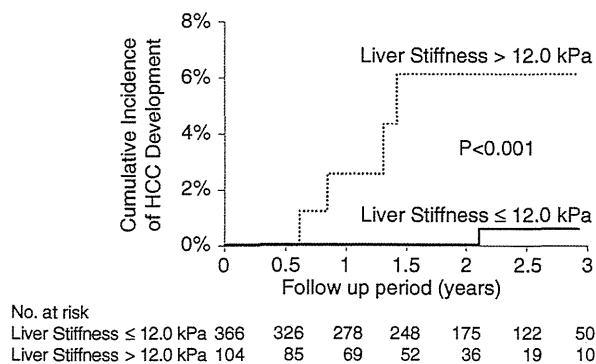
Factors related to HCC development were examined, and univariate analysis extracted elevated AST, elevated AFP and liver stiffness of more than 12.0 kPa as significant factors, and multivariate analysis revealed that liver stiffness of more than 12.0 kPa was an independent factor. A history of interferon 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





**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  $\leq$ 12 kPa is shown. Four and one case developed HCC among cases with liver stiffness  $>$ 12 kPa and  $\leq$ 12 kPa, respectively. Liver stiffness  $>$ 12 kPa was associated with a significantly higher risk of HCC development than liver stiffness  $\leq$ 12 kPa ( $P < 0.001$ ). No case with liver stiffness  $\leq$ 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,<sup>17,21–23</sup> and 10.3–17.5 kPa with sensitivity ranging 92–100% and specificity 88–97% in non-alcoholic fatty liver disease cases.<sup>8,11,24</sup> 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.<sup>16,18,25</sup>

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.<sup>25</sup> 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.<sup>13,16</sup> 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.<sup>6,26,27</sup> 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.<sup>6,28–30</sup> 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.<sup>31–33</sup> 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 <i>n</i> = 5	Patients who did not develop HCC <i>n</i> = 465	Univariate			Multivariate		
			HR	95% CI	<i>P</i>	HR	95% CI	<i>P</i>
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 <sup>9</sup> /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.<sup>34</sup> 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.<sup>15</sup> 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,<sup>32</sup> 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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**Original Article**

# Deep sequencing analysis of variants resistant to the non-structural 5A inhibitor daclatasvir in patients with genotype 1b hepatitis C virus infection

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**Aim:** Daclatasvir, a non-structural (NS)5A 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 HCV 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 (11.8%) and

34 (30.9%) of the 110 patients, respectively, and significantly more frequently than in the control plasmid. Simultaneous L31M/V/F and Y93H mutations were detected in four of the 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 ( $P = 0.042$ ).

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

**Key words:** deep sequencing, hepatitis C virus, 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 60–80% by combination with pegylated (PEG) interferon (IFN)/ribavirin (RBV) therapy.<sup>1</sup> However, high SVR rates following combination therapy have not been seen in null-responders to previous PEG IFN/RBV combination therapy.<sup>2</sup> Under these circumstances, development of more effective drug therapies with less serious adverse effects is anticipated.

Daclatasvir (BMS-790052), a non-structural (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.<sup>3</sup> Moreover, among all NS5A inhibitors, daclatasvir is most advanced in its development for clinical use.<sup>4,5</sup> 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.<sup>6,7</sup>

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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 for IFN-based therapy.<sup>8,9</sup> 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.<sup>8-11</sup> 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.<sup>12-14</sup> 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.

## 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: (i) negative for hepatitis B surface antigen; (ii) no other forms of hepatitis, such as primary biliary cirrhosis, autoimmune liver disease or alcoholic liver disease; (iii) free of co-infection with HIV; and (iv) 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

Hepatitis C virus RNA extraction, complementary DNA synthesis, amplification by two-step nested polymerase chain reaction (PCR) from serum samples using primers specific for partial viral regions and direct sequencing were carried out as described previously.<sup>15,16</sup> Generated sequence files were assembled using Vector NTI software (Invitrogen, Tokyo, Japan) and base-calling errors were corrected following inspection of the chromatogram.

This direct sequencing procedure was performed to determine the dominant viral sequences of the core,<sup>17</sup> the IFN sensitivity-determining region (ISDR)<sup>18</sup> and the IFN-ribavirin resistance determining region (IRRDR)<sup>19</sup> from the serum of each patient.

### IL28B SNP analysis

Recent reports have disclosed a significant correlation between polymorphisms in the IL28B gene and patients' responses to PEG IFN plus RBV therapy for HCV.<sup>20-22</sup> Human genomic DNA was extracted from peripheral blood using a blood DNA extraction kit (QIAGEN, Tokyo, Japan) according to the manufacturer's protocol. The genotyping of each DNA sample was performed by real-time PCR with a model 7500 sequencer (ABI, Tokyo, Japan) using FAM- and VIC-labeled single nucleotide (nt) polymorphism (SNP) probes for the locus rs8099917 (ABI).

### Deep sequencing

Deep sequencing of part of the viral NS5A region was performed for each of the 110 patients. Briefly, RNA was extracted from the stored sera and reverse transcribed to complementary DNA.<sup>23</sup> Then, two-step nested PCR was carried out with primers specific for the NS5A region of the HCV genome. To avoid PCR selection bias, we



**Table 1** Patient characteristics classified by their responses to previous PEG IFN/RBV combination therapy

	Naïve n = 59	Relapser n = 30	Null responder n = 21	P
Age (years)	62.3 ± 11.5	62.7 ± 9.1	61.2 ± 7.7	0.719
Sex F/M	35/24	16/14	9/12	0.427
AST (IU/L)	35.4 ± 12.6	43.9 ± 53.4	45.3 ± 14.6	0.008
ALT (IU/L)	34.6 ± 18.5	45.3 ± 73.2	51.8 ± 23.5	<0.001
PLT ( $\times 10^{-4}/\mu\text{L}$ )	15.1 ± 5.6	14.3 ± 3.8	13.8 ± 4.8	0.582
Alb (g/dl)	4.2 ± 0.4	4.3 ± 0.3	4.2 ± 0.5	0.334
$\gamma$ -GT (IU/L)	35.2 ± 37.7	37.6 ± 45.1	67.1 ± 55.2	<0.001
AFP (ng/mL)	5.7 ± 6.3	4.5 ± 3.6	14.7 ± 29.0	<0.001
Core a.a. 70 R	35 (59.3%)	23 (76.7%)	6 (28.6%)	0.003
Core a.a. 91 L	41 (69.5%)	18 (60.0%)	14 (66.7%)	0.672
ISDR 2-	14 (23.7%)	5 (16.7%)	2 (9.5%)	0.340
IRRDR 5-	29 (49.2%)	13 (43.3%)	8 (38.1%)	0.181
IL28B SNP TT	38 (64.4%)	27 (90.0%)	6 (25.6%)	<0.001

$\gamma$ -GT,  $\gamma$ -glutamyltransferase; a.a., amino acid; AFP,  $\alpha$ -fetoprotein; Alb, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; IRRDR, interferon-ribavirin resistance determining region; ISDR, interferon sensitivity-determining region; PEG IFN/RBV, pegylated interferon/ribavirin; PLT, platelets.

searched for the most conserved DNA sequence regions around NS5A by examining sequence information published previously from 43 HCV positive individuals from Japan<sup>16</sup> and designed novel primers for this study (Supplementary Table 1). This PCR procedure amplified 436 viral nt, including the 1st to 432nd nt of the NS5A region. The primers for the second-round PCR had barcodes, 10 nt in length, attached and these differed for each sample, so that the PCR products from each sample were identifiable. After the band densities of the PCR products were quantified using a Pico Green dsDNA Assay Kit (Invitrogen), the concentrations of the samples were adjusted to a common value and pooled samples were prepared.

Libraries were then subjected to emulsion PCR, the enriched DNA beads were loaded onto a picotiter plate and pyrosequencing was carried out with a Roche GS Junior/454 sequencing system using titanium chemistry (Roche, Branford, CT, USA). The Roche Variant Analyzer version 2.5pl (Roche) was used for the analysis.

### Statistical analysis

Statistical differences in the parameters, including all available patients' demographic, biochemical, hematological, virological and SNP data in the three groups (naïve, relapser and null responder), classified according to the response to previous PEG IFN/RBV therapy, were determined using the  $\chi^2$ -test for categorical variables and Kruskal-Wallis test for numerical variables. Statistical differences in the parameters in two groups

(Y93H positive, Y93H negative) were determined by Student's *t*-test or Mann-Whitney *U*-test for numerical variables and Fisher's exact test or  $\chi^2$ -test for categorical variables. Variables that achieved statistical significance ( $P < 0.05$ ) in univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. We also calculated the odds ratios and 95% confidence intervals. All *P*-values of less than 0.05 by the two-tailed test were considered significant.

## RESULTS

### Average read numbers obtained by deep sequencing and the background error rate

TO PERFORM DEEP sequencing analysis of the NS5A region from many patients, simultaneous analysis was carried out using the barcode primers and approximately 3826 reads were obtained per sample from each group of patients (naïve, relapser and null responder) (Table 2). Because a previous clinical phase II study had yielded a significantly high SVR rate, especially in the patients with a null response to previous PEG IFN/RBV combination therapy, we classified the patients according to their responses to previous PEG IFN/RBV combination therapy with the assumption that differences in the response to PEG IFN/RBV might influence the daclatasvir response.

The background error rate of pyrosequencing was calculated with a plasmid containing a cloned HCV sequence (pCV-J4L6S)<sup>24</sup> and the read number for the



**Table 2** Amplicon read numbers obtained by deep sequencing

	<i>n</i>	Average reads $\pm$ SD (range)/sample
Naïve	59	3603.9 $\pm$ 1758.4 (655–10 293)
Relapser	30	3980.4 $\pm$ 3295.9 (445–14 330)
Null responder	21	4601.6 $\pm$ 2385.5 (1187–9579)
Plasmid	7	5448.3 $\pm$ 1299.1 (2277–7000)

SD, standard deviation.

plasmid is also shown in Table 2. Though seven runs of the plasmid produced 2277–7000 reads, with an average of 5448 reads, there was no background error at amino acid (a.a.) 31, 32 or 93 in NS5A. Because the background error rate was 0% at each position, the presence of mutations at 0.1% or higher was considered to be significant, based on the 95% confidence interval (0–0.1%) calculated for 0% in 2227 reads. The background error rate coincided almost exactly with the background error rate obtained in our recent study.<sup>23</sup>

### Baseline characteristics

The baseline characteristics of the 110 patients are shown in Table 1. The data for viral factors (core a.a. 70, core a.a. 91, NS5A-ISDR and NS5A-IRRDR) in the table were obtained by direct sequencing as described in Methods. As shown in the table, there were significant differences among the three groups in aspartate aminotransferase, alanine aminotransferase,  $\gamma$ -glutamyltransferase,  $\alpha$ -fetoprotein, core a.a. 70 and IL28B SNP (rs8099917). Meanwhile, there was no significant difference in background factors of age and sex or factors associated with liver fibrosis such as platelets and albumin.

### Detection of NS5A-resistance mutations by deep sequencing

Because previous reports showed that L31M/V/F, P32L and Y93H are resistance mutations in NS5A of genotype 1b HCV, the presence of these mutations was analyzed by deep sequencing. Table 3 shows the rate of NS5A resistance mutations at a.a. 31, 32 and 93. At a.a. 32, no mutation was found in any of the 110 patients. Regarding a.a. 31, resistance mutations (L31M/V/F) were observed in 13 of the 110 patients (11.8%) and, despite no significant difference, tended to occur more frequently in the relapser group and naïve group than in the null group. Meanwhile, the a.a. 93 resistance mutation (Y93H) was observed in 34 of the 110 patients (30.9%) and, despite no significant difference, also tended to occur more frequently in the naïve and relapser groups than in the null group. Simultaneous a.a. 93 and 31 resistance mutations were observed in only four of 110 patients (3.6%) and these four patients all belonged to the naïve group. More detailed deep sequence results for the four patients with simultaneous mutation of L31M/V/F and Y93H are shown in Table 2. Although the substitution rate of L31M/V/F in these patients was low, all isolates with L31M/V/F also featured the Y93H change.

### Mutation rates of L31M/V/F and Y93H in each patient

Figure 1 show the mutation rates of L31M/V/F and Y93H in each patient. One bar indicates the resistance mutation rate in one patient, obtained by deep sequencing. It was found that minor viral populations that were not detected by direct sequencing could be detected by deep sequencing.

**Table 3** Presence of daclatasvir-resistance amino acid substitutions in daclatasvir treatment-naïve patients, determined by deep sequencing

	Naïve <i>n</i> = 59	Relapser <i>n</i> = 30	Null responder <i>n</i> = 21	Naïve vs relapser <i>P</i>	Naïve vs null <i>P</i>	Relapser vs Null <i>P</i>
L31M/V/F %, median (range)†	2.0 (0.0–99.8)	4.1 (0.0–100.0)	0.2 (0.0–3.4)	0.895	0.295	0.317
Pts with L31M/V/F (%)‡	8 (13.6%)	4 (13.3%)	1 (4.8%)	1.000	0.510	0.612
P32L %, median (range)†	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	1.000	1.000	1.000
Pts with P32L (%)‡	0 (0%)	0 (0%)	0 (0%)	1.000	1.000	1.000
Y93H %, median (range)†	11.7 (0.0–99.1)	7.9 (0.0–100.0)	4.1 (0.0–45.3)	0.824	0.190	0.301
Pts with Y93H (%)‡	21 (35.6%)	10 (33.3%)	3 (14.3%)	1.000	0.112	0.224

†Median proportion per patient (Pts).

‡Number of Pts with the mutant.

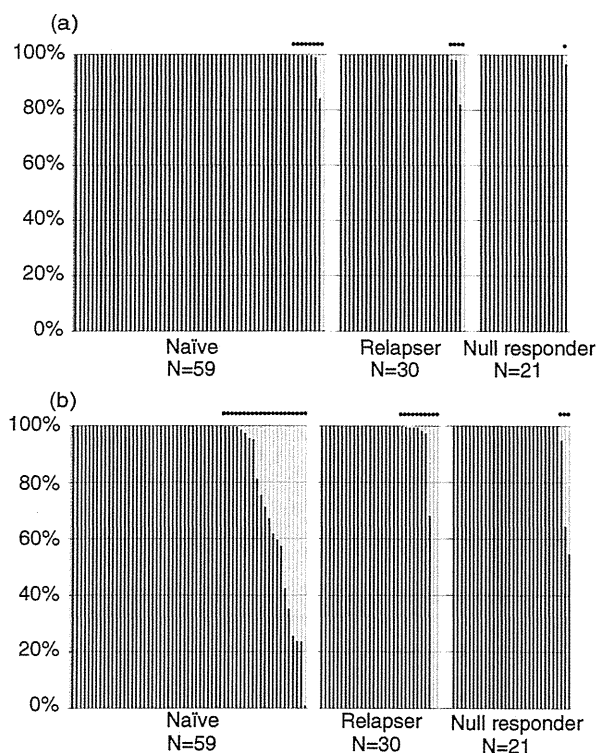


Figure 1 Percentage of mutations in the non-structural (NS)5A region associated with resistance to daclatasvir is presented, classified by the response to previous pegylated interferon/ribavirin (PEG IFN/RBV) therapy (naïve, null responder and relapser). (a) NS5A amino acid (a.a.) 31, (b) NS5A a.a. 93. Each bar indicates the mutation rate in one patient and a dot above a bar shows a patient with a mutation detected by deep sequencing.

In order to compare our deep sequencing data with previous direct sequencing data in terms of the frequency of NS5A mutations, the notion of "cut-offs" was introduced into our deep sequencing data, assuming that direct sequencing could detect minor populations existing above those cut-off levels. When the cut-off level of 50% was defined to detect minor populations by direct sequencing, L31M/V/F mutations and the Y93H mutations were detected in 1.8% (2/110 patients) and 7.3% (8/110) of our patients, respectively, while the values became 1.8% (2/110 patients) and 15.4% (15/110) when 20% was defined as the cut-off level. These results are comparable to the mutation rate determined previously by direct sequencing and that found in the database.<sup>25</sup>

### Univariate and multivariate analysis of factors related to the NS5A Y93H mutation

Focusing on the Y93H mutation that is found most frequently in daclatasvir treatment-naïve patients, clinical background factors that would determine efficacy of PEG IFN/RBV combination therapy patients were investigated by univariate analysis of their association with the Y93H substitution (Table 4). Three factors, the IL28B SNP, core a.a. 70 and IRRDR, were found to be correlated with the Y93H substitution with statistical significance in the univariate analysis. In patients with the Y93H mutation, the major type (TT) was frequently observed as the IL28B SNP, while arginine (R) was frequently observed at core a.a. 70 and the number of substitutions in the IRRDR was higher. There was no significant difference in the number of mutations in the ISDR but that number tended to be higher in patients with the Y93H mutation, similar to the IRRDR.

The IL28B SNP, core a.a. 70 and IRRDR, which were correlated significantly with the a.a. 93 mutation by univariate analysis, were subjected to multivariate analysis (Table 4). The IL28B SNP major type (TT) was extracted as an independent significant factor with the odds ratio of 3.67 ( $P=0.042$ ). The mutation rates of L31M/V/F and Y93H in each patient, classified by the IL28B SNP, are presented in Figure 2. Y93H mutations were found significantly more frequently in IL28B TT patients than that in IL28B non-TT patients.

### DISCUSSION

**I**N THIS STUDY, viral mutations conferring resistance to the NS5A replication complex inhibitor daclatasvir were investigated by deep sequencing in daclatasvir treatment-naïve genotype 1b HCV patients and the mutations, especially Y93H, were detected more frequently than predicted by direct sequencing. Interestingly and importantly, the presence of the Y93H mutation correlated with the IL28B SNP of the host, suggesting the possibility that IL28B major type patients who may show a favorable response to IFN have a greater risk of being infected by daclatasvir-resistant HCV.

Regarding the daclatasvir-resistance mutations L31M/V/F, P32L and Y93H in genotype 1b HCV, it has been reported that a single mutation produces 5–28-fold increased resistance and simultaneous mutations of L31M/V/F and Y93H yield 10 989–21 674-fold increased resistance in genotype 1b HCV infection.<sup>6</sup> Previously, the frequencies of L31M/V/F and Y93H were reported to be 2.7% and 8.2%, respectively, with direct

**Table 4** Univariate and multivariate analysis of factors associated with non-structural (NS)5A-Y93H

Variables	No. of patients	NS5A-Y93H substitution		Univariate analysis (n = 110)		Multivariate analysis (n = 110)	
		Positive (n = 34)	Negative (n = 76)	Odds ratio (95% CI)	P-value	Odds ratio (95% CI)	P-value
Age, ≥65 years	48	16 (47.1%)	32 (42.1%)	1.22 (0.54–2.76)	0.629		
Sex male	50	16 (47.1%)	34 (44.7%)	1.10 (0.49–2.47)	0.821		
AST, ≥41 IU/L	38	11 (32.4%)	27 (35.5%)	0.87 (0.37–2.05)	0.746		
ALT, ≥41 IU/L	33	9 (26.5%)	24 (31.6%)	0.78 (0.32–1.92)	0.590		
Platelets, ≤12 × 10 <sup>4</sup> /mm <sup>3</sup>	35	12 (35.3%)	23 (30.3%)	1.43 (0.61–3.33)	0.601		
Alb, ≤4 g/dL	25	9 (26.5%)	16 (21.1%)	0.69 (0.28–1.70)	0.422		
γ-GT, ≥41 IU/L	30	10 (29.4%)	20 (26.3%)	1.25 (0.51–3.08)	0.628		
AFP, ≥10	16	5 (14.7%)	11 (14.5%)	1.02 (0.32–3.20)	0.974		
IL28B TT	71	29 (85.3%)	42 (55.3%)	4.70 (1.64–13.43)	0.004	3.67 (1.05–12.88)	0.042
Core a.a. 70 R	64	25 (73.5%)	39 (51.3%)	2.64 (1.09–6.38)	0.032	1.19 (0.40–3.55)	0.759
Core a.a. 91 L	73	24 (70.6%)	49 (64.5%)	1.32 (0.55–3.17)	0.531		
ISDR, † ≥2	21	8 (23.5%)	13 (17.1%)	1.49 (0.55–4.02)	0.430		
IRRDR, ‡ ≥5	54	23 (67.5%)	32 (42.1%)	2.88 (1.23–6.73)	0.015	2.37 (0.98–5.74)	0.056
NS5A L31M/V/F positive	11	2 (5.9%)	9 (11.8%)	0.46 (0.10–2.28)	0.345		
History of IFN therapy	59	21 (61.8%)	38 (50.0%)	1.62 (0.71–3.69)	0.255		

†ISDR mutation number.

‡IRRDR mutation number.

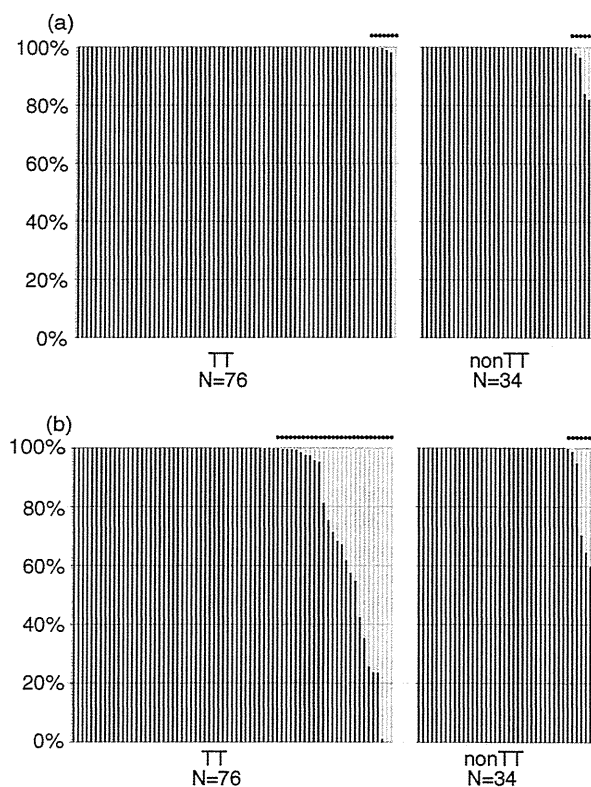
γ-GT, γ-glutamyltransferase; a.a., amino acid; AFP, α-fetoprotein; Alb, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CI, confidence interval; IRRDR, interferon-ribavirin resistance determining region; ISDR, interferon sensitivity-determining region; PEG IFN/RBV, pegylated interferon/ribavirin; PLT, platelets.

sequencing in genotype 1b daclatasvir treatment-naïve Japanese patients ( $n = 294$ ) and this was comparable with the frequency (3.8% and 8.3%, respectively) in genotype 1b patients, determined from the European HCV database ( $n = 1796$ ).<sup>6,25</sup> Among the regimens including daclatasvir for genotype 1b HCV infection, until now, only the result of a phase II trial of daclatasvir/asunaprevir therapy for 43 patients has been reported.<sup>9,9</sup> In that study, the pretreatment presence of HCV carrying Y93H was significantly associated with non-SVR to that regimen and, moreover, that viruses carrying mutations in both regions of NS5A (L31M/V/F and Y93H) and of NS3 (D168A/V) emerged in most of the non-SVR patients after virological failure.

In our study, the presence of L31M/V/F and Y93H mutations in daclatasvir treatment-naïve genotype 1b patients was comparable to a previous study which involved direct sequencing, when a cut-off value was introduced to our deep sequencing data, although the prevalence of NS5A mutants changed depending on the cut-off value. However, deep sequencing analysis revealed that NS5A L31M/V/F and Y93H mutations were detectable in 13 (11.8%) and 34 of the 110 (30.9%) patients, respectively, above the background error rate of 0.1% and significantly more frequently than

detected by direct sequencing. These results demonstrate that deep sequencing is useful for the detection of viral mutants present as minor variants.

Do HCV populations with Y93H present as minor variants have any association with clinical characteristics? Interestingly, univariate analysis based on the relationship between the presence of the Y93H variant and clinical factors or factors determining treatment efficacy to PEG IFN/RBV combination therapy extracted three significant factors: the IL28B SNP, core a.a. 70 and the IRRDR (Table 4). All these factors were associated with a favorable response to PEG IFN/RBV combination therapy in the group with the Y93H-resistance mutation.<sup>26</sup> Despite that the difference did not reach statistical significance, the number of substitutions in the ISDR also tended to be higher in the group with the Y93H mutation, similar to the IRRDR. It was quite intriguing that multivariate analysis of the presence of Y93H extracted the IL28B major type, the SNP was significantly associated with favorable IFN responses, as an independent factor (Table 4). On the other hand, because it is known that the IL28B SNP is closely linked with core a.a. 70, it is assumed that core 70R should be observed more frequently in the group with Y93H.<sup>16</sup>



**Figure 2** Percentage of mutations at the non-structural (NS)5A region for resistance to daclatasvir is presented, classified by the IL28B SNP (TT or non-TT). (a) NS5A amino acid (a.a.) 31, (b) NS5A a.a. 93. Each bar indicates the mutation rate in one patient and a dot above a bar shows a patient with a mutation detected by deep sequencing.

Then, do NS5A resistant variants with Y93H that are present prior to treatment affect the response to daclatasvir treatment? At present, in genotype 1b infection, daclatasvir is scheduled to be used in combination with other DAA but not with IFN. Considering the correlation between IL28B SNP and Y93H, and the fact that Y93H variants may be sensitive to IFN but resistant to daclatasvir,<sup>27</sup> patients with the IL28B major type may be recommended to receive IFN-based therapy rather than DAA regimens including daclatasvir, because those patients have a greater chance of being infected with daclatasvir-resistant Y93H variants leading to treatment failure. In contrast, the IL28B minor type patients who have poor responses to IFN may be more promising candidates.

The true clinical influence of Y93H on treatment responses remain unknown and further elucidation is

mandatory after the approval of daclatasvir for clinical use. In particular, it is important to clarify the cut-off values as to the mixture ratio of Y93H to Y93 wild type in establishing clinical resistance, if the presence of viruses with Y93H before treatment really does affect the response. If so, it is also important to clarify whether the proportion of Y93H variants changes during the clinical course (the natural course or during therapy including IFN) in order to determine the most appropriate timing for introducing daclatasvir. However, it is possible for Y93H variants to disappear after IFN treatment considering that Y93H variants may be sensitive to IFN. The mechanism of the relationship between the IL28B SNP and Y93H also is not clear at present. Considering that wild-type NS5A is known to be associated in its ISDR region with IFN resistance and with the IL28B minor SNP (TG/GG),<sup>28</sup> it is possible that wild-type NS5A Y93 also is associated with IFN resistance and with IL28B minor SNP, although further elucidation is necessary.

We acknowledge that the PCR technique has a risk of producing biased amplicons according to the PCR primer sequences and therefore we designed novel primers in this study by searching for the most conserved sequence regions around NS5A. We speculate that the sequence bias might have been avoided at least to some extent considering the fact that the NS5A mutation rate in this study was quite compatible with that of a previous study and that obtained from the public database.

In conclusion, we detected by deep sequencing the substantial presence of resistance mutations to daclatasvir, Y93H in particular, in daclatasvir treatment-naïve patients and these were not detectable by direct sequencing. We also showed that IL28B major type patients who have favorable responses to IFN may have a higher risk of being infected with Y93H HCV than IL28B minor type patients, suggesting that those patients may have a higher risk of developing daclatasvir resistance, although further studies are needed.

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