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.

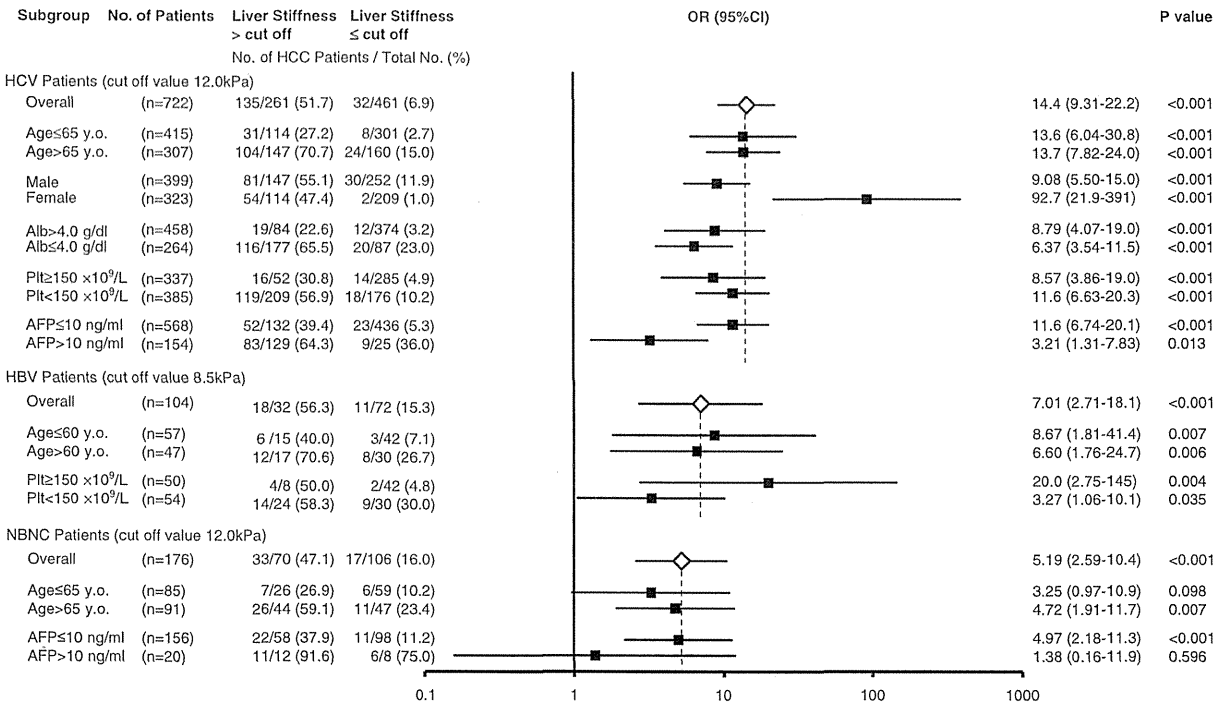


Figure 2 Odds ratio (OR) for the presence of hepatocellular carcinoma (HCC) in specified subgroups associated with liver stiffness over the cut-off value. The OR (95% confidence interval [CI]) for HCC and a *P*-value are shown for each subgroup of hepatitis C virus (HCV) patients with liver stiffness >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.

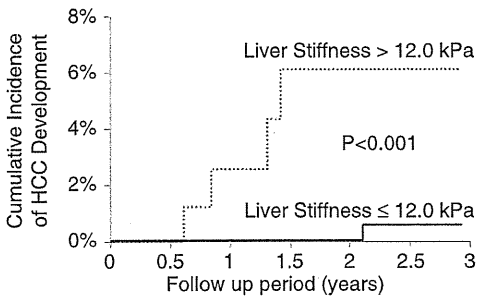
follow-up period of 691 days. The incidence of HCC development was significantly higher among cases with liver stiffness of more than 12.0 kPa than among those with liver stiffness of 12.0 kPa or less ($P < 0.001$, by log–rank test) (Fig. 3).

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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Article Type: Original Article

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Received date: 26/11/2013

Accepted date: 14/02/2014

Revised date: 13/02/2014

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

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

Financial disclosure:

This study was supported in part by a grant-in-aid from the Ministry of Education, Science, Sports and Culture of Japan (23390195, 23791404, 24590964 and 24590965), and in part by a grant-in-aid from the Ministry of Health, Labour, and Welfare of Japan (H23-kanen-001, H23-kanen-004, H23-kanen-006, H24-kanen-002, H24-kanen-004 and H25-kanen-006).

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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

HCV RNA extraction, complementary DNA synthesis, amplification by two-step nested PCR from serum samples using primers specific for partial viral regions and direct sequencing were carried out as described previously [15, 16]. 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 [17], the interferon sensitivity-determining region (ISDR) [18] and the interferon-ribavirin resistance determining region (IRRDR) [19] from the serum of each patient.

IL28B SNP analysis

Recent reports have disclosed a significant correlation between polymorphisms in the interleukin (IL) 28B gene and patients' responses to pegylated-IFN plus ribavirin therapy for HCV [20-22]. 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 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 [23]. 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 searched for the most conserved DNA sequence regions around NS5A by examining sequence information published previously from 43 HCV-positive individuals from Japan [16] and designed novel primers for this study (Supplementary Table1). This PCR procedure amplified 436 viral nucleotides, including the 1st to 432nd nucleotide of the NS5A region. The primers for the second-round PCR had barcodes, 10 nucleotides (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). 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-square 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 the Student t test or Mann-Whitney's U test for numerical variables and Fisher's exact

probability test or Chi-square 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 <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 2 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) [24] and the read number for the plasmid is also shown in Table 2. Though seven runs of the plasmid produced 2,277-7,000 reads, with an average of 5,448 reads, there was no background error at amino acid (aa) 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 2,227 reads. The background error rate coincided almost exactly with the background error rate obtained in our recent study [23].

Baseline characteristics

The baseline characteristics of the 110 patients are shown in Table 1. The data for viral factors (core aa 70, core aa 91, NS5A-ISDR and NS5A-IRRDR) in the table were obtained by direct sequencing as described in ~~the~~ Patients and Methods. As shown

in the table, there were significant differences among the three groups in AST, ALT, γGTP, alpha-fetoprotein, core aa 70, and IL28B SNP (rs8099917). Meanwhile, there was no significant difference in background factors of age and gender or liver fibrosis associated factors such as PLT and Alb.

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 aa 31, 32, and 93. At aa 32, no mutation was fond in any of the 110 patients. Regarding aa 31, resistance mutations (L31M/V/F) were observed in 13/110 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 aa 93 resistance mutation (Y93H) was observed in 34/110 (30.9%) and, despite no significant difference, also tended occur more frequently in the naïve group and relapser group than in the null group. Simultaneous aa 93 and 31 resistance mutations were observed in only 4/110 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 Supplementary 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 1A and B 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.

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 [25].

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 aa 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 aa 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 aa 70, and IRRDR, which were correlated significantly with the aa 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 IL28 SNP, are presented in Figure 2A and B. Y93H mutations were found significantly more frequently in IL28B TT patients than that in IL28B non-TT patients.

DISCUSSION

In 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 might 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- to 28-fold increased resistance and simultaneous mutations of L31M/V/F and Y93H yield 10,989 to 21,674-fold increased resistance in genotype 1b HCV infection [6]. Previously, the frequencies of L31 M/V/F and Y93H were reported to be 2.7% and 8.2%, respectively, with direct 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) [6, 25]. 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 [8, 9]. In that study, the pretreatment presence of HCVs 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 non-SVR patients after virological failure.

In our study, the presence of L31 M/V/F and Y93H mutations in