infection by different viral genotypes. *Hepatology* 2010; 52: 33–7.
25 Yu ML, Huang CF, Huang JF *et al.* Role of interleukin-28B polymorphism in the treatment of hepatitis C virus genotype 2 infection in Asian patients. *Hepatology* 2011; 53: 7–13.
26 Thomas DL, Thio CL, Martin MP *et al.* Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* 2009; 461: 798–801.
27 Yang M, Rao HY, Feng B, Zhang W, Wei L. The impact of IL28B polymorphisms on spontaneous clearance of hepatitis C virus infection: a meta-analysis. *J Gastroenterol Hepatol* 2013; 28: 1114–21.
28 Hadziyannis SJ, Sette H Jr, Morgan TR *et al.* Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann Intern Med* 2004; 140: 346–55.
29 McHutchison JG, Lawitz EJ, Shiffman ML *et al.* Peginterferon alfa-2b or alfa-2a with ribavirin for treatment of hepatitis C infection. *N Engl J Med* 2009; 361: 580–93.
30 Lok AS, Everhart JE, Wright EC *et al.* Maintenance peginterferon therapy and other factors associated with hepatocellular carcinoma in patients with advanced hepatitis C. *Gastroenterology* 2011; 140: 840–9.
31 Fried MW, Shiffman ML, Reddy KR *et al.* Peginterferon alpha-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; 347: 975–82.
32 McHutchison JG, Lawitz EJ, Shiffman ML *et al.* Peginterferon alfa-2b with ribavirin for treatment of hepatitis C infection. *N Engl J Med* 2009; 361: 580–93.
33 Bota S, Sporea I, Sirli R *et al.* Severe adverse events during antiviral therapy in hepatitis C virus cirrhotic patients: a systematic review. *World J Hepatol* 2013; 27: 120–6.
34 Buti M, Valdés A, Sánchez-Avila F, Esteban R, Lurie Y. Extending combination therapy with peginterferon alfa-2b plus ribavirin for genotype 1 chronic hepatitis C late responders: a report of 9 cases. *Hepatology* 2003; 37: 1226–7.
35 Berg T, von Wagner M, Nasser S *et al.* Extended treatment duration for hepatitis C virus type 1: comparing 48 weeks versus 72 weeks of peginterferon-alfa-2a plus ribavirin. *Gastroenterology* 2006; 130: 1086–97.
36 Sanchez-Tapias JM, Diago M, Escartin P *et al.* Peginterferon-alfa-2a plus ribavirin for 48 versus 72 weeks in patients with detectable hepatitis C virus RNA at week 4 of treatment. *Gastroenterology* 2006; 131: 451–60.
37 Akuta N, Suzuki F, Hirakawa M *et al.* A matched case-controlled study of 48 and 72 weeks of peginterferon plus ribavirin combination therapy in patients infected with HCV genotype 1b in Japan: amino acid substitution in HCV core region as predictor of sustained virological response. *J Med Virol* 2009; 81: 452–8.
38 Poordad F, Lawitz E, Kowdley KV *et al.* Exploratory study of oral combination antiviral therapy for hepatitis C. *N Engl J Med* 2013; 368: 45–53.
39 Zeuzem S, Soriano V, Asselah T *et al.* Faldaprevir and deleobuvir for HCV genotype 1 infection. *N Engl J Med* 2013; 369: 630–9.
40 Gane EJ, Stedman CA, Hyland RH *et al.* Nucleotide polymerase inhibitor sofosbuvir plus ribavirin for hepatitis C. *N Engl J Med* 2013; 368: 34–44.
41 Suzuki Y, Ikeda1 K, Suzuki F *et al.* Dual oral therapy with daclatasvir and asunaprevir for patients with HCV genotype 1b infected and limited treatment options. *J Hepatol* 2013; 58: 655–62.

Hepatology Research

Hepatology Research 2014; 44: 983–992 doi: 10.1111/hepr.12280

Original Article

Interleukin 28B polymorphism predicts interferon plus ribavirin treatment outcome in patients with hepatitis C virus-related liver cirrhosis: A multicenter retrospective study in Japan

Satoshi Shakado,[1,2] Shotaro Sakisaka,[1,2] Takeshi Okanoue,[3] Kazuaki Chayama,[4] Namiki Izumi,[5] Joji Toyoda,[6] Eiji Tanaka,[7] Akio Ido,[8] Tetsuo Takehara,[9] Kentaro Yoshioka,[10] Yoichi Hiasa,[11] Hideyuki Nomura,[12] Masataka Seike,[13] Yoshiyuki Ueno[14] and Hiromitsu Kumada[15]

[1]*Department of Gastroenterology and Medicine,* [2]*Division of Advanced Clinical Research for Viral Hepatitis and Liver Cancer, Faculty of Medicine, Fukuoka University, Fukuoka,* [3]*Saiseikai Suita Hospital,* [9]*Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, Suita,* [4]*Department of Gastroenterology and Metabolism, Applied Life Sciences, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima,* [5]*Department of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Musashino,* [6]*Department of Gastroenterology and Hepatology, Sapporo Kousei Hospital, Sapporo,* [7]*Department of Medicine, Shinshu University School of Medicine, Matsumoto,* [8]*Department of Gastroenterology and Hepatology, Kagoshima University, Kagoshima,* [10]*Department of Liver, Biliary Tract and Pancreas Diseases, Fujita Health University, Toyoake,* [11]*Department of Gastroenterology and Metabology, Ehime University Graduate School of Medicine, Touon,* [12]*The Center for Liver Disease, Shin-Kokura Hospital, Kitakyushu,* [13]*Department of Gastroenterology, Faculty of Medicine, Oita University, Yufu,* [14]*Department of Gastroenterology, Yamagata University, Yamagata, and* [15]*Department of Hepatology, Toranomon Hospital, Kawasaki, Japan*

Aim: This study evaluated the efficacy of interferon plus ribavirin and examined whether interleukin 28B (IL28B) polymorphism influenced treatment outcome in Japanese patients with hepatitis C virus (HCV)-related liver cirrhosis (LC).

Methods: Fourteen collaborating centers provided details of 261 patients with HCV-related LC undergoing treatment with interferon plus ribavirin. Univariate and multivariate analyses were used to establish which factors predicted treatment outcome.

Results: Eighty-four patients (32.2%) achieved a sustained virological response (SVR). SVR rates were 21.6% (41/190) in patients with HCV genotype 1 with high viral load (G1H) and 60.6% (43/71) in patients with non-G1H. In patients with non-G1H, treatment outcome was effective irrespective of IL28B polymorphism. In those with G1H, SVR was achieved in 27.1% of patients with the IL28B rs8099917 TT allele compared with 8.8% of those with the TG/GG alleles ($P = 0.004$). In patients with G1H having TT allele, treatments longer than 48 weeks achieved significantly higher SVR rates than treatments less than 48 weeks (34.6% vs 16.4%, $P = 0.042$). In patients with G1H having TG/GG alleles, treatments longer than 72 weeks achieved significantly higher SVR rates than treatments less than 72 weeks (37.5% vs 4.1%, $P = 0.010$).

Conclusion: Interferon plus ribavirin treatment in Japanese patients with non-G1H HCV-related LC was more effective than those with G1H and not influenced by IL28B polymorphism. In those with G1H, IL28B polymorphism may predict SVR and guide treatment duration: SVR rates were higher in those with the TT allele treated for more than 48 weeks and those with the TG/GG alleles treated for more than 72 weeks.

Key words: cirrhosis, hepatitis C virus, interferon, interleukin 28B, ribavirin

Correspondence: Dr Satoshi Shakado, Department of Gastroenterology and Medicine, Faculty of Medicine, Fukuoka University, 7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan. Email: shakado @cis.fukuoka-u.ac.jp

Received 29 August 2013; revision 15 November 2013; accepted 17 November 2013.

INTRODUCTION

CHRONIC HEPATITIS C virus (HCV) infection is a leading cause of liver cirrhosis worldwide.[1] Patients with HCV-related liver cirrhosis (LC) are at increased risk of hepatic decompensation and hepatocellular

carcinoma (HCC).[2–4] The therapeutic goal in these patients should be the prevention of liver-related mortality. A randomized trial conducted in Japan was the first to suggest that interferon (IFN) may reduce the risk of HCC in patients with HCV-related LC.[5] Recent studies have shown that patients with HCV-related LC who achieved a sustained virological response (SVR) with antiviral therapy had a significant reduction in liver-related mortality.[6,7] However, patients with HCV-related LC show a lower SVR rate than non-cirrhotic patients, as well as a reduced tolerance to the therapy.[8,9] A previous meta-analysis revealed that the overall SVR rate in patients with cirrhosis was 33.3%, and was significantly higher in patients with HCV genotypes 2 and 3 (55.4%) than in those with HCV genotypes 1 and 4 (21.7%).[10]

Genome-wide association studies have recently shown that single nucleotide polymorphisms (SNP) near the interleukin 28B (IL28B) region (rs8099917, rs12979860) are the most powerful predictors of SVR to pegylated (PEG) IFN plus ribavirin in patients with HCV genotype 1 infection.[11–13] However, it is not clear whether IL28B polymorphism can be used to predict the virological response to treatment of HCV-related LC. This study evaluated the efficacy of IFN plus ribavirin, and the association between IL28B polymorphism and the treatment efficacy in Japanese patients with HCV-related LC.

METHODS

THIS WAS A multicenter retrospective study of patients with HCV-related LC who had received treatment with IFN plus ribavirin in 14 hospitals in Japan.

Patient selection

Data were collected from 290 patients with HCV-related LC receiving treatment with IFN plus ribavirin in 14 academic and community hospitals. All patients had compensated HCV-related LC with clinical or histological data available. The diagnosis of cirrhosis met at least one of the following criteria: liver biopsy specimens with cirrhosis, diffuse formation of the nodules on the liver surface in peritoneoscopy, over 12.5 kPa in liver stiffness values on transient elastography, signs of portal hypertension on ultrasound scan (splenomegaly, portal vein enlargement, re-permeabilization of the umbilical vein, or presence of portal-systemic shunts), presence of esophageal varices on endoscopy or positive values using the following discriminant by Ikeda and colleagues: $z = 0.124 \times (\gamma\text{-globulin [\%]}) + 0.001 \times$ (hyaluronate) ($\mu g\ L^{-1}$) $- 0.075 \times$ (platelet count [$\times 10^4$ counts/mm^3]) $- 0.413 \times$ sex (male, 1; female, 2) $- 2.005$.[14–16] Principal investigators in 14 hospitals identified eligible patients and entered data in a predefined database.

Combination therapy

Of the 290 patients identified, 29 were not genotyped for IL28B SNP, thus the data of 261 patients were analyzed. A total of 190 patients were infected with HCV genotype 1 with high viral load (>100 KIU/mL) (G1H) (72.8%) and the remaining 71 (27.2%) were classified as non-G1H. Twenty-two patients were HCV genotype 1 with low viral load, 46 were genotype 2a or 2b, and three were of unknown genotype. Two hundred and twenty-four (85.8%) patients were treated with PEG IFN-α-2b (1.5–1.0 μg/kg bodyweight per week), 20 (7.7%) patients were treated with PEG IFN-α-2a (45–180 μg/week) and the remaining 17 (6.5%) patients were treated with IFN-α-2b or IFN-β. IFN-α-2b and IFN-β were administrated at a median dose of 6 million units each day (seven times per week for the initial 2 or 4 weeks, followed by three times per week thereafter). All patients also received oral ribavirin (600–1000 mg/day). Median treatment duration was 48 and 28 weeks in G1H and non-G1H, respectively. The individual attending physician determined the treatment regimes and their duration.

Virological response during therapy and definitions

The efficacy end-point was SVR, defined as undetectable serum HCV RNA 24 weeks after treatment. Relapse was defined as undetectable serum HCV RNA at the last treatment visit but detectable serum HCV RNA again at the last follow-up visit. Breakthrough was defined as reappearance of serum HCV RNA during treatment. A non-responder was defined as serum HCV RNA never undetectable during treatment. A rapid virological response (RVR) was defined as undetectable serum HCV RNA at treatment week 4, and a complete early virological response (cEVR) was defined as undetectable serum HCV RNA at treatment week 12. A late virological response (LVR) was defined as detectable serum HCV RNA at 12 weeks that became undetectable within 36 weeks of the start of treatment.

Determination of IL28B genotype

Interleukin 28B (rs8099917) was genotyped in each of the 14 hospitals by Invader assay, TaqMan assay or by direct sequencing, as previously described.[17,18]

Statistical analysis

Results were analyzed on the intention-to-treat principle. Mean differences were tested using Student's *t*-test. The difference in the frequency distribution was analyzed with Fisher's exact test. Univariate and multivariate logistic regression analyses were used to identify factors independently associated with SVR. The odds ratios (OR) and 95% confidence intervals (95% CI) were also calculated. The parameters that achieved statistical significance on univariate analysis were entered into multivariate logistic regression analysis to identify significant independent factors. Data were analyzed with JMP version 9.0 for Macintosh (SAS Institute, Cary, NC, USA). All statistical analyses were two sided, and $P < 0.05$ was considered significant.

RESULTS

OF THE 261 patients included in our analysis, 84 patients (32.2%) achieved SVR (Fig. 1). The rate of relapse and breakthrough was 24.9% and the non-responder rate was 33.3%. There were 25 patients (9.6%) who required early discontinuation of treatment because of adverse events. Baseline demographic and clinical features are summarized in Table 1. The age of the patients was 60.7 ± 8.9 years and 50.6% were male. Of the patients studied, 125 patients (47.9%) had been treated with IFN previously, and 75 (28.7%) had not responded to previous treatment. One hundred and six patients (40.6%) had been treated for HCC before. There were 85 patients with esophageal varices (32.6%). There were 190 patients with G1H and 133 (70%) of these had the TT allele at IL28B rs8099917. There were 71 patients in the non-G1H group, 51 (71.8%) of whom were found to have the TT allele at IL28B rs8099917.

Virological response rates in patients with G1H and non-G1H HCV-related LC

The SVR rates were 21.6% (41/190) in patients with G1H and 60.6% (43/71) in patients with non-G1H (Table 2). There were no statistically significant differences between the G1H and non-G1H groups with regard to dose reduction rates of IFN or ribavirin. Dose reduction of IFN was required in 51.3% of patients and dose reduction of ribavirin in 53.6% of patients. Treatment duration in patients in the G1H group was significantly longer than those in the non-G1H group ($P = 0.010$).

Association between IL28B rs8099917 genotype and treatment response

Sustained virological response was achieved in 37.0% of patients with the rs8099917 TT allele and 20.8% in those with the TG or GG allele. Virological responses, including SVR, relapse and breakthrough, in patients with the rs8099917 TT allele were significantly higher than in those with rs8099917 TG or GG allele ($P = 0.013$ and 0.012, respectively; Table 3). The proportion of non-responders among patients with the rs8099917 TG or GG allele was significantly higher than in those with the TT allele ($P = 0.002$). There was no

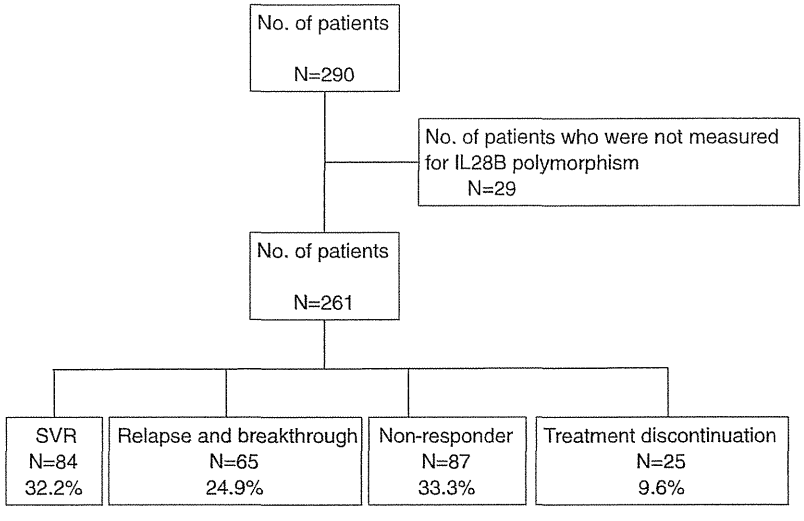

Figure 1 Flowchart showing the characteristics of the study cohort. IL28B, interleukin 28B; SVR, sustained virological response.

Table 1 Summary of demographic and baseline characteristics ($n = 261$)

	G1H, $n = 190$	Other than G1H, $n = 71$	All patients, $n = 261$
Sex (M : F)	95:95	37:34	132:129
Age (years)	60.5 ± 9.3	61.2 ± 7.8	60.7 ± 8.9
BMI (kg/m^2)	23.8 ± 3.5	23.4 ± 3.2	23.7 ± 3.4
IFN treatment history	91 (47.9%)	34 (47.9%)	125 (47.9%)
HCC treatment history	75 (39.5%)	31 (43.7%)	106 (40.6%)
Presence of EV	60 (31.6%)	25 (35.2%)	85 (32.6%)
Total bilirubin (mg/dl)	1.1 ± 0.9	1.1 ± 1.4	1.1 ± 1.2
AST (IU/L)	79.1 ± 44.2	75.8 ± 57.7	79.9 ± 52.7
ALT (IU/L)	82.4 ± 56.4	81.9 ± 75.4	83.3 ± 66.2
GGT (IU/L)	83.8 ± 107.8	87.0 ± 140.1	84.6 ± 115.8
Albumin (g/dL)	3.7 ± 0.5	3.8 ± 0.4	3.7 ± 0.5
Prothrombin (%)	86.2 ± 14.4	83.7 ± 16.7	85.5 ± 15.1
WBC (/μL)	4407 ± 1592	4190 ± 1930	4348 ± 1667
Hemoglobin (g/dL)	13.2 ± 1.8	13.1 ± 1.8	13.1 ± 1.8
Platelets (10^4/mm^3)	11.8 ± 6.7	11.8 ± 6.3	11.8 ± 6.6
AFP (ng/mL)	48.9 ± 224.7	24.0 ± 29.3	45.4 ± 193.9
DCP (mAU/mL)	66.8 ± 372.3	155.3 ± 620.4	92.4 ± 450.8
IL28B (TT : TG + GG)	133:57	51:20	184:77

All values are expressed as mean ± standard deviation.
AFP, α-fetoprotein; ALT, alanine transaminase; AST, aspartate aminotransferase; BMI, body mass index; DCP, des-γ-carboxy prothrombin; EV, esophageal varices; G1H, genotype 1 with high viral load; GGT, γ-glutamyltransferase; HCC, hepatocellular carcinoma; IFN, interferon; IL28B, interleukin 28B rs8099917 genotype; WBC, white blood cell.

significant association between the IL28B genotype and the incidence of adverse events.

Among patients in the G1H group, SVR was achieved in 27.1% (36/133) of those with the TT allele and 8.8% (5/57) of those with the TG or GG allele (Table 4). There was no statistically significant difference between IL28B genotype and viral response in patients with non-G1H.

Table 2 Summary of treatment and sustained virological response rates ($n = 261$)

	G1H, $n = 190$	Other than G1H, $n = 71$	All patients, $n = 261$
Dose reduction of IFN	$n = 98$ (51.6%)	$n = 36$ (50.7%)	$n = 134$ (51.3%)
Dose reduction of RBV	$n = 107$ (56.3%)	$n = 33$ (46.5%)	$n = 140$ (53.6%)
Treatment duration (weeks)			
Mean ± SD	45.3 ± 21.6	37.7 ± 19.6	43.2 ± 21.4
Median	48	28	48
SVR	$n = 41$ (21.6%)	$n = 43$ (60.6%)	$n = 84$ (32.2%)

G1H, genotype 1 with high viral load; IFN, interferon; RBV, ribavirin; SD, standard deviation; SVR, sustained virological response.

Table 3 Association between IL28B rs8099917 polymorphism and treatment response in 261 hepatitis C virus-related liver cirrhotic patients

IL28B	TT ($n = 184$)	TG + GG ($n = 77$)	*P*-value
SVR	68 (37.0%)	16 (20.8%)	0.013
Relapse and breakthrough	54 (29.3%)	11 (14.3%)	0.012
Non-responder	44 (23.9%)	43 (55.8%)	0.002
Discontinuation	18 (9.8%)	7 (9.1%)	1.000

IL28B, interleukin 28B rs8099917 genotype; SVR, sustained virological response.

Table 4 Sustained virological response associated between IL28B rs8099917 polymorphism and G1H in hepatitis C virus-related liver cirrhosis patients

IL28B	TT ($n = 184$)	TG + GG ($n = 77$)	*P*-value
G1H	36/133 (27.1%)	5/57 (8.8%)	0.004
Other than G1H	32/51 (62.7%)	11/20 (55.0%)	0.596

G1H, genotype 1 with high viral load; IL28B, interleukin 28B rs8099917 polymorphism.

Predictive factors associated with SVR

Differences in the characteristics of patients with SVR and those in whom SVR was not achieved are summarized in Table 5. Neither age, sex, alanine transaminase, aspartate aminotransferase, prothrombin activity, hemoglobin nor platelet counts appeared to significantly influence the chance of achieving SVR. The patients who achieved SVR had a lower body mass index, higher white blood cell count and higher serum albumin than those who did not, and were more likely to have non-G1H and the TT allele of IL28B rs8099917. Multivariate analysis identified that possession of the IL28B rs8099917 TT allele (OR = 2.85; 95% CI, 1.01–9.15; $P = 0.047$) and non-G1H (OR = 6.49; 95% CI, 1.77–26.43; $P = 0.005$) as significant determinants of SVR.

Treatment duration and efficacy in patients with G1H

Of the patients with G1H, 79 (41.6%) received less than 48 weeks of treatment. The number receiving 48–52 weeks, 53–72 weeks, over 72 weeks and unknown duration of treatment were 54 (28.4%), 41 (21.6%), 14 (7.4%) and two (1.1%), respectively. The median duration of treatment in patients who achieved RVR and cEVR was 48 weeks, but was significantly longer (66 weeks) in those with an LVR ($P < 0.001$). Table 6 shows the SVR rates of those with different IL28B genotypes and on-treatment viral response. The SVR rate in patients who achieved LVR was significantly lower than those who achieved RVR and cEVR ($P = 0.002$). Of the patients with G1H found to have the IL28B TG or GG genotype, none achieved RVR and only two achieved cEVR.

Predictors of SVR in patients with G1H and the TT allele

Patients with G1H and the TT allele who achieved SVR had higher platelet counts, higher serum albumin and had undergone over 48 weeks of treatment. Multivariate analysis identified platelet count (OR = 1.08; 95% CI, 1.01–1.18; $P = 0.047$), serum albumin (OR = 2.78; 95% CI, 1.14–7.42; $P = 0.031$) and over 48 weeks of treatment duration (OR = 2.53; 95% CI, 1.07–6.49; $P = 0.042$) as significant determinants of SVR (Table 7).

Predictors of SVR in patients with G1H and the TG or GG allele

Patients who had G1H and the TG or GG allele who achieved SVR had a higher total dose of ribavirin ($P = 0.011$) and more than 72 weeks of treatment duration ($P = 0.010$).

Treatment tolerability and adverse events

Table 8 illustrates details of the patients who experienced adverse events higher than grade 2. There were

Table 5 Factors associated with sustained virological response in hepatitis C virus-related liver cirrhosis patients

Factors	SVR (+), ($n = 84$)	SVR (–), ($n = 177$)	*P*-value	Multivariate analyses		
				Odds ratio	95% CI	*P*-value
BMI (kg/m^2)	22.9 ± 3.5	24.0 ± 3.3	0.019			
WBC (/μL)	4727 ± 2096	4168 ± 1376	0.013			
Albumin (g/dL)	3.83 ± 0.48	3.68 ± 0.46	0.018			
Other than G1H	$n = 43$ (51.2%)	$n = 28$ (15.8%)	<0.001	6.49	1.77–26.43	0.005
IL28B TT	$n = 68$ (81.0%)	$n = 116$ (65.5%)	0.012	2.85	1.01–9.15	0.047

P-values were obtained by logistic regression model.
BMI, body mass index; CI, confidence interval; G1H, genotype 1 with high viral load; IL28B, interleukin 28B rs8099917 polymorphism; SVR, sustained virological response; WBC, white blood cell.

Table 6 Sustained viral response rates between IL28B genotype and on-treatment viral response in the patients with G1H

	IL28B TT	IL28B TG/GG	All patients
RVR	7/7	0/0	7/7
	100%	0%	100%
cEVR	15/26	1/2	16/28
	57.7%	50%	57.1%
LVR	14/44	4/11	18/55
	31.8%	36.4%	32.7%

cEVR, complete early virological response (defined as serum HCV RNA negative at treatment week 12); G1H, genotype 1 with high viral load; HCV, hepatitis C virus; IL28B, interleukin 28B rs8099917; LVR, late virological response (defined as serum HCV RNA detectable at 12 weeks and undetectable at 36 weeks after the start of treatment); RVR, rapid virological response (defined as serum HCV RNA negative at treatment week 4).

two cases of liver decompensation, two cases of interstitial pneumonia, one case of cerebral hemorrhage and one case of cerebral infarction. The cause of death in two patients was decompensation of LC. In one patient, treatment was stopped after 4 weeks, and in another, treatment was stopped after 32 weeks because of hepatic failure. The IFN dose was reduced in 134 patients (51.3%), and the ribavirin dose was reduced in 140 patients (53.6%) and discontinued in 60 patients (23.0%). Among patients who had treatment discontinued, 27 patients (10.3%) had treatment withdrawn because of no virological response and 33 patients (12.6%) because of severe adverse events. In patients in whom treatment was discontinued, three patients had SVR and five had a relapse.

IL28B alleles predicting SVR in G1H group

The influence of IL28B rs8099917 genotype on SVR in G1H is shown in Figure 2. Overall, there were 84 patients (32.2%) who achieved SVR with IFN plus ribavirin in HCV-related LC. The SVR was 60.6% in those with non-G1H, and was not significantly influenced by IL28B rs8099917 genotype (the SVR in TT patients was 62.7% compared with 55.0% in TG or GG patients). In contrast, in patients with G1H, the SVR of patients with IL28B rs8099917 genotype TT was significantly higher than those with rs8099917 TG or GG (27.1% vs 8.8%, $P = 0.004$). In patients with G1H and IL28B TT, the SVR of those treated for over 48 weeks was significantly higher than those treated for less than 48 weeks (34.6% vs 16.4%, $P = 0.042$). In patients with G1H and IL28B TG/GG, the SVR of those treated for over 72 weeks was significantly higher than those treated for less than 72 weeks (37.5% vs 4.1%, $P = 0.010$).

Table 8 Adverse events higher than grade 2

	No. of patients (%)
Anemia	63 (24.1%)
Thrombocytopenia	31 (11.9%)
Leukopenia	19 (7.3%)
Rash and itching	17 (6.5%)
Fatigue and general malaise	15 (5.7%)
Gastrointestinal disorders	5 (1.9%)
Depression	5 (1.9%)
Development of hepatocellular carcinoma	3 (1.1%)
Respiratory disorders	3 (1.1%)
Liver decompensation	2 (0.8%)
Malignant neoplasm	2 (0.8%)
Interstitial pneumonia	2 (0.8%)
Cerebral hemorrhage	1 (0.4%)
Cerebral infarction	1 (0.4%)
Cholangitis	1 (0.4%)
Retinal hemorrhage	1 (0.4%)
Diabetes decompensation	1 (0.4%)
Palpitation	1 (0.4%)

DISCUSSION

WE FOUND THAT in Japanese patients with G1H HCV-related LC, the likelihood of achieving SVR with IFN plus ribavirin combination therapy was influ-

Table 7 Factors associated with sustained virological response in the patients with G1H and TT allele of IL28B rs8099917 ($n = 133$)

Factors	SVR (+) ($n = 36$)	SVR (−) ($n = 97$)	*P*-value	Multivariate analyses		
				Odds ratio	95% CI	*P*-value
Platelets (10^4/mm^3)	14.5 ± 11.5	10.6 ± 4.2	0.024	1.08	1.01–1.18	0.047
Albumin (g/dL)	3.92 ± 0.50	3.69 ± 0.46	0.018	2.78	1.14–7.42	0.031
Treatment duration, over 48 weeks	$n = 27$ (75%)	$n = 51$ (52.6%)	0.023	2.53	1.07–6.49	0.042

P-values were obtained by logistic regression model.
CI, confidence interval; G1H, genotype 1 with high viral load; IL28B, interleukin 28B; SVR, sustained virological response.

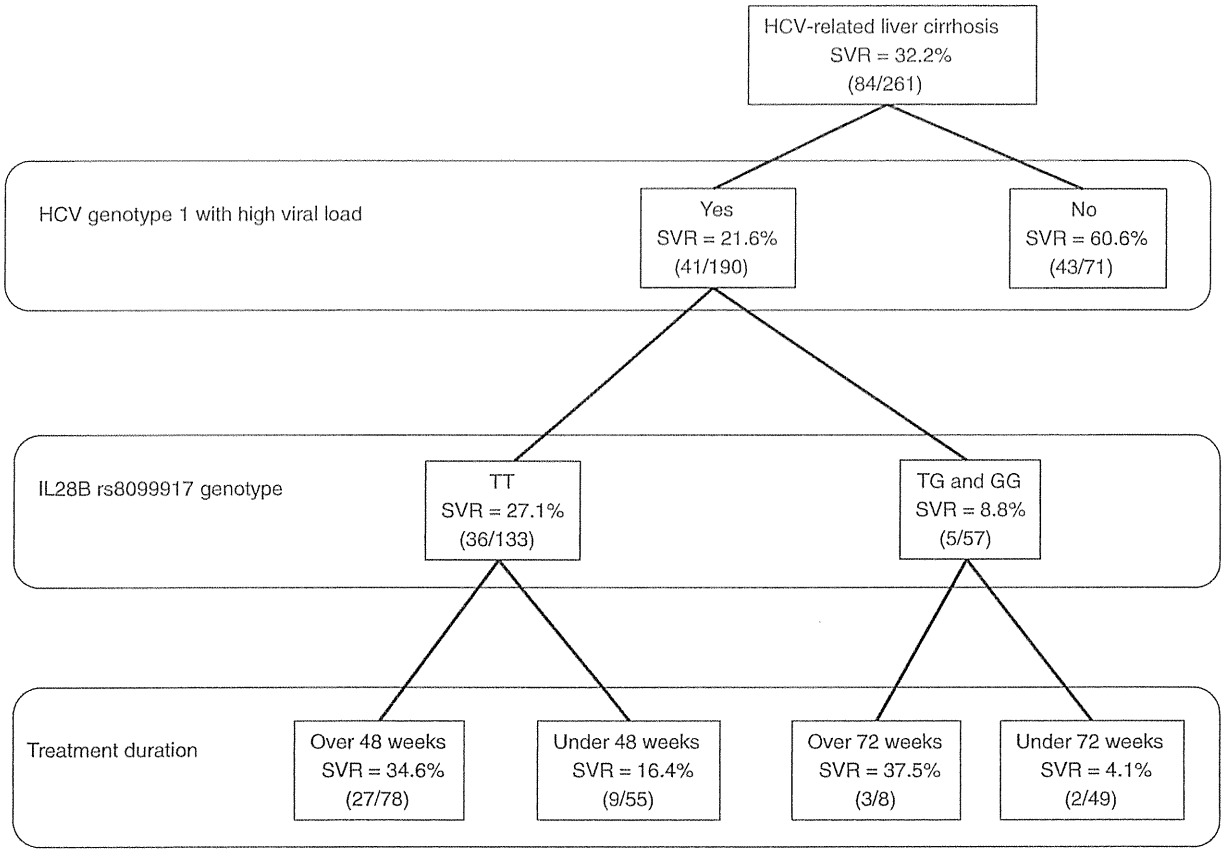

Figure 2 SVR in HCV-related liver cirrhosis patients treated with interferon plus ribavirin. In patients with G1H and the IL28B TT allele, the SVR rate of those who were treated for over 48 weeks was significantly higher than those treated for less than 48 weeks ($P = 0.042$). In patients with G1H and IL28B TG/GG, the SVR rate of patients treated for over 72 weeks was significantly higher than those treated for less than 72 weeks ($P = 0.010$). G1H, genotype 1 with high viral load; HCV, hepatitis C virus ; IL28B, interleukin 28B rs8099917; SVR, sustained virological response.

enced by a polymorphism at IL28B rs8099917. In contrast, SVR rates in non-G1H were higher than those in G1H, irrespective of IL28B genotype. This is the first report to demonstrate that an IL28B polymorphism can influence SVR rate in patients treated with IFN plus ribavirin combination therapy for G1H HCV-related LC. These results suggest that HCV genotypes, viral load and IL28B polymorphism should be taken into when determining antiviral therapy for HCV-related LC. In patients with HCV-related LC, IL28B genotyping may be a useful tool to determine the best antiviral therapy.

Recently, host genetic variation near the IL28B on chromosome 19, which encodes IFN-λ-3, have been shown to be associated with SVR to PEG IFN plus ribavirin in patients infected with HCV genotype 1.[11–13] Although some investigators have shown that IL28B polymorphisms are associated with a favorable response to treatment in patients with non-1 genotype infection, the association between the variants in IL28B and SVR in non-1 genotype-infected patients remains controversial.[19–25] IL28B polymorphisms are also a strong predictive factor for spontaneous HCV clearance.[26,27] However, the precise mechanism associated with the action of IL28B polymorphisms has not been fully elucidated.

Pegylated IFN plus ribavirin combination therapy has become the standard of care treatment for chronic HCV infection. The SVR rates range 42–46% in patients with HCV genotype 1 or 4 infection and 76–82% in patients with HCV genotype 2 or 3 infection, respectively.[9,28,29] However, in patients with HCV-related LC the SVR rate is even lower than in non-LC patients, reflecting reduced

tolerance to the therapy.[8–10] Although patients with HCV-related LC are difficult to treat, patients who achieved SVR showed a lower rate of liver-related adverse outcomes and improved survival.[8–10] Moreover, a randomized controlled trial showed that patients with HCV-related LC who received long-term PEG IFN treatment had a lower risk of HCC than controls.[30] Thus, IFN treatment for HCV-related LC is an effective means of preventing HCC, irrespective of whether SVR is achieved. In this study, the SVR was very low in patients with G1H and the TG or GG allele. Therefore, for these patients, long-term administration of maintenance IFN should be considered to reduce the risk of developing of HCC even if SVR is unlikely to be achieved.

Patients with advanced liver disease have a higher rate of adverse events when taking IFN and ribavirin combination therapy than patients with mild disease. Adverse events, such as neutropenia, thrombocytopenia and anemia, often require dose reduction of IFN or ribavirin. Previous studies have demonstrated that in patients with HCV-related LC, the rate of dose reductions in IFN and ribavirin range 6.9–20.6% and 16.7–27.1%, respectively.[31–33] In our study, IFN and ribavirin dose reductions were needed in 51.3% and 53.6% of patients, respectively. These are higher than those reported in other studies, but the discontinuation rate was slightly lower (12.6%).[33] Many patients required reductions in the doses of IFN and/or ribavirin early in the treatment period because of adverse events, but ultimately were able to tolerate long-term administration. It might be safer to start low-dose antiviral therapy with IFN plus ribavirin in HCV-related LC and titrating the dose upward as tolerated with the aim of long-term treatment, rather than beginning with the full dose and risking adverse events that would curtail antiviral therapy.

In patients infected with HCV genotype 1, previous studies have demonstrated that SVR rates of late virological responders (HCV RNA detectable at 12 weeks and undetectable at 24 weeks after the start of treatment) could be improved when treatment was extended to 72 weeks, compared with the standard treatment duration of 48 weeks, largely as a result of reducing post-treatment relapse rates.[34–37] In this study, the SVR rate in patients who had an LVR was significantly lower than those who achieved RVR or cEVR. However, the duration of treatment in the patients with a LVR was significantly longer than those who achieved cEVR or RVR. Individual physicians determined the duration of treatment based on the time at which serum HCV RNA became undetectable, accounting for the improved SVR rates in those receiving extended courses. Nevertheless, the safety and effectiveness of more than 48 weeks of antiviral therapy in patients with HCV-related LC has not been examined. We found that patients with the IL28B rs8099917 genotype TT, treatment of more than 48 weeks achieved a higher SVR rate than treatment of less than 48 weeks, and in those with the TG or GG alleles SVR rates were greater in those who received more than 72 weeks of treatment. The response to treatment is a very important guide of treatment duration in HCV-related LC. Further prospective studies using larger numbers of patients matched for race, HCV genotype, viral load and treatment durations would be required to explore the relationships between IL28B polymorphism and the treatment response to combination therapy in patients with HCV-related LC.

Recently, new trials of IFN-free combination therapy with direct-acting antivirals (DAA) such as protease-inhibitor, non-structural (NS)5A inhibitor or NS5B polymerase inhibitor nucleotide analog have shown a strong antiviral activity against HCV.[38–40] A previous study reported that the IL28B genotype can affect the response to an IFN-free regimen, but this result has been unclear in other regimens.[38–40] In a study of Japanese patients with HCV genotype 1b infection, dual oral DAA therapy (NS5A inhibitor and NS3 protease inhibitor) without IFN achieved an SVR rate of 90.5% of 21 patients with no response to previous therapy and in 63.6% of 22 patients who had been ineligible for treatment with PEG IFN.[41] However, lack of a virological response to DAA was also seen in patients with no response or partial response to previous therapy. In these patients with viral resistance to DAA, the combination therapy with IFN and DAA may be a means of eliminating HCV, and IL28B genotyping may be a useful tool in determining the best antiviral therapy and duration of treatment.

This study had certain limitations. Selection bias cannot be excluded, considering the retrospective nature of the work. However, all patients had well-established cirrhosis and had received IFN plus ribavirin in hepatitis centers throughout Japan. Our patients received a variety of IFN treatments (IFN-α, IFN-β and PEG IFN), several different doses of IFN and ribavirin, and several treatment durations. In the intention-to-treat analysis, the overall SVR rate was 32.2%; in patients with G1H it was 21.6% but was 60.6% in those with non-G1H. Interestingly, the overall SVR rate in this study was similar to that found in previous studies of patients with advanced fibrosis or cirrhosis treated with IFN or PEG IFN plus ribavirin.[8–10] Thus, although there were some

limitations, our findings contribute to providing valuable information to guide clinical decisions.

In conclusion, the combination therapy with IFN plus ribavirin in Japanese patients with non-G1H HCV-related LC was more effective than those with G1H and not influenced by IL28B polymorphism. However, in patients with G1H, IL28B polymorphism may be a strong predictive factor for SVR. Extending treatment may provide a better outcome in those with the IL28B TT allele treated for more than 48 weeks and in those with the TG/GG alleles treated for more than 72 weeks.

ACKNOWLEDGMENT

THIS STUDY WAS supported by a Grant-in-Aid from the Japanese Ministry of Health, Welfare, and Labor.

REFERENCES

1 Niederau C, Lange S, Heintges T *et al.* Progress of chronic hepatitis C: results of a large, prospective cohort study. *Hepatology* 1998; **28**: 1687–95.
2 Fattovich G, Giustina G, Degos F *et al.* Morbidity and mortality in compensated cirrhosis type C; a retrospective follow up study of 384 paatients. *Gastroenterology* 1997; **112**: 463–72.
3 Hu KQ, Tong MJ. The long-term outcomes of patients with compensated hepatitis C virus-related cirrhosis and history of parenteral exposure in the United States. *Hepatology* 1999; **29**: 1311–16.
4 Sangionvanni A, Prati GM, Fasani P *et al.* The natural history of compensated cirrhosis due to hepatitis C virus: a 17-year cohort study of 214 patients. *Hepatology* 2006; **43**: 1303–10.
5 Nishiguchi S, Kuroki T, Nakatani S *et al.* Randomized trail of effects of interferon-alpha on incidence of hepatocellular carcinoma in chronic active hepatitis C with cirrhosis. *Lancet* 1995; **346**: 1051–5.
6 Shiratori Y, Ito Y, Yokosuka O *et al.* Antiviral therapy for cirrhotic hepatitis C: association with reduced hepatocellular carcinoma development and improved. *Ann Int Med* 2005; **142**: 105–14.
7 Bruno S, Stroffolini T, Colombo M *et al.* Sustained virological response to interferon-alpha is associated with improved outcome in HCV related cirrhosis: a retrospective study. *Hepatology* 2007; **45**: 579–87.
8 Wright TL. Treatment of patients with hepatitis C and cirrhosis. *Hepatology* 2002; **36**: S185–194.
9 Manns MP, McHutchison JG, Gordon SC *et al.* Peginterferon alpha-2b plus ribavirin compared with interferon aalpha-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomized trial. *Lancet* 2001; **358**: 958–65.
10 Bota S, Sporea I, Popescu A *et al.* Response to standard of care antiviral treatment in patients with HCV liver cirrhosis – a systematic review. *J Gastrointestin Liver Dis* 2011; **20**: 293–8.
11 Ge D, Fellay J, Thompson AJ *et al.* Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* 2009; **461**: 399–401.
12 Tanaka Y, Nishida N, Sugiyama M *et al.* Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet* 2009; **41**: 1105–9.
13 Suppiah V, Moldovan M, Ahlenstiel G *et al.* IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat Genet* 2009; **41**: 1100–4.
14 Ziol M, Handra-Luca A, Kettaneh A *et al.* Noninvasive assessment of liver fibrosis by measurement of stiffness in patients with chronic hepatitis C. *Hepatology* 2005; **41**: 48–54.
15 Castera L, Vergniol J, Foucher J *et al.* Prospective comparison of transient elastography, fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. *Gastroenterology* 2005; **128**: 343–50.
16 Ikeda K, Saitoh S, Kobayashi M *et al.* Distinction between chronic hepatitis and liver cirrhosis in patients with hepatitis C virus infection. Practical discriminant function using common laboratory data. *Hepatol Res* 2000; **18**: 252–66.
17 Ohnishi Y, Tanaka T, Ozaki K, Yamada R, Suzuki H, Nakamura Y. A high-throughput SNP typing system for genome-wide association studies. *J Hum Genet* 2001; **46**: 471–7.
18 Suzuki A, Yamada R, Chang X *et al.* Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginie deminase 4, are associated with rheumatoid arthritis. *Nat Genet* 2003; **34**: 395–402.
19 Kawaoka T, Hayes CN, Ohishi W *et al.* Predictive value of the IL28B polymorphism on the effect of interferon therapy in chronic hepatitis C patients with genotype 2a and 2b. *J Hepatol* 2010; **54**: 408–14.
20 Sarrazin C, Susser S, Doehring A *et al.* Importance of IL28B gene polymorphisms in hepatitis C virus genotype 2 and 3 infected patients. *J Hepatol* 2010; **54**: 415–21.
21 Asselah T, De Muynck S, Broët P *et al.* IL28B polymorphism is associated with treatment response in patients with genotype 4 chronic hepatitis C. *J Hepatol* 2012; **56**: 527–32.
22 De Nicola S, Aghemo A, Rumi MG *et al.* Interleukin 28B polymorphism predicts pegylated interferon plus ribavirin treatment outcome in chronic hepatitis C genotype 4. *Hepatology* 2012; **55**: 336–42.
23 Akuta A, Suzuki F, Seko Y *et al.* Association of IL28B genotype and viral response of hepatitis C virus genotype 2 to interferon plus ribavirin combination therapy. *J Med Virol* 2012; **84**: 1593–9.
24 Montes-Cano MA, Garcia-Lozano JR, Abad-Molina C *et al.* Interleukin-28B genetic variants and hepatitis virus

infection by different viral genotypes. *Hepatology* 2010; **52:** 33–7.
25 Yu ML, Huang CF, Huang JF *et al.* Role of interleukin-28B polymorphism in the treatment of hepatitis C virus genotype 2 infection in Asian patients. *Hepatology* 2011; **53:** 7–13.
26 Thomas DL, Thio CL, Martin MP *et al.* Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* 2009; **461:** 798–801.
27 Yang M, Rao HY, Feng B, Zhang W, Wei L. The impact of IL28B polymorphisms on spontaneous clearance of hepatitis C virus infection: a meta-analysis. *J Gastroenterol Hepatol* 2013; **28:** 1114–21.
28 Hadziyannis SJ, Sette H Jr, Morgan TR *et al.* Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann Intern Med* 2004; **140:** 346–55.
29 McHutchison JG, Lawitz EJ, Shiffman ML *et al.* Peginterferon alfa-2b or alfa-2a with ribavirin for treatment of hepatitis C infection. *N Engl J Med* 2009; **361:** 580–93.
30 Lok AS, Everhart JE, Wright EC *et al.* Maintenance peginterferon therapy and other factors associated with hepatocellular carcinoma in patients with advanced hepatitis C. *Gastroenterology* 2011; **140:** 840–9.
31 Fried MW, Shiffman ML, Reddy KR *et al.* Peginterferon alpha-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; **347:** 975–82.
32 McHutchison JG, Lawitz EJ, Shiffman ML *et al.* Peginterferon alfa-2b with ribavirin for treatment of hepatitis C infection. *N Engl J Med* 2009; **361:** 580–93.
33 Bota S, Sporea I, Sirli R *et al.* Severe adverse events during antiviral therapy in hepatitis C virus cirrhotic patients: a systematic review. *World J Hepatol* 2013; **27:** 120–6.
34 Buti M, Valdés A, Sánchez-Avila F, Esteban R, Lurie Y. Extending combination therapy with peginterferon alfa-2b plus ribavirin for genotype 1 chronic hepatitis C late responders: a report of 9 cases. *Hepatology* 2003; **37:** 1226–7.
35 Berg T, von Wagner M, Nasser S *et al.* Extended treatment duration for hepatitis C virus type 1: comparing 48 weeks versus 72 weeks of peginterferon-alfa-2a plus ribavirin. *Gastroenterology* 2006; **130:** 1086–97.
36 Sanchez-Tapias JM, Diago M, Escartin P *et al.* Peginterferon-alfa-2a plus ribavirin for 48 versus 72 weeks in patients with detectable hepatitis C virus RNA at week 4 of treatment. *Gastroenterology* 2006; **131:** 451–60.
37 Akuta N, Suzuki F, Hirakawa M *et al.* A matched case-controlled study of 48 and 72 weeks of peginterferon plus ribavirin combination therapy in patients infected with HCV genotype 1b in Japan: amino acid substitution in HCV core region as predictor of sustained virological response. *J Med Virol* 2009; **81:** 452–8.
38 Poordad F, Lawitz E, Kowdley KV *et al.* Exploratory study of oral combination antiviral therapy for hepatitis C. *N Engl J Med* 2013; **368:** 45–53.
39 Zeuzem S, Soriano V, Asselah T *et al.* Faldaprevir and deleobuvir for HCV genotype 1 infection. *N Engl J Med* 2013; **369:** 630–9.
40 Gane EJ, Stedman CA, Hyland RH *et al.* Nucleotide polymerase inhibitor sofosbuvir plus ribavirin for hepatitis C. *N Engl J Med* 2013; **368:** 34–44.
41 Suzuki Y, Ikeda1 K, Suzuki F *et al.* Dual oral therapy with daclatasvir and asunaprevir for patients with HCV genotype 1b infected and limited treatment options. *J Hepatol* 2013; **58:** 655–62.

Hepatology Research

Hepatology Research 2014; 44: 141–148 doi: 10.1111/hepr.12214

Review Article

Application of deep sequence technology in hepatology

Masashi Ninomiya,[1] Yoshiyuki Ueno[2,3] and Tooru Shimosegawa[1]

[1]*Division of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai,* [2]*Department of Gastroenterology, and* [3]*CREST, Yamagata University Faculty of Medicine, Yamagata, Japan*

Deep sequencing technologies are currently cutting edge, and are opening fascinating opportunities in biomedicine, producing over 100-times more data compared to the conventional capillary sequencers based on the Sanger method. Next-generation sequencing (NGS) is now generally defined as the sequencing technology that, by employing parallel sequencing processes, producing thousands or millions of sequence reads simultaneously. Since the GS20 was released as the first NGS sequencer on the market by 454 Life Sciences, the competition in the development of the new sequencers has become intense. In this review, we describe the current deep sequencing systems and discuss the application of advanced technologies in the field of hepatology.

Key words: deep sequencing technology, next-generation sequencing, pyrosequencing, sequencing-by-synthesis

INTRODUCTION

DEEP SEQUENCING TECHNOLOGIES are currently hot topics and are opening fascinating opportunities in the study of biomedicine. They can produce over 100-times more data compared to the traditional capillary sequencers based on the standard Sanger method. This technology was introduced in *Nature Methods* as the method of the year in 2007.[1]

In 1975, Sanger first reported the sequencing method by primed synthesis with DNA polymerase.[2] In 1977, epoch-making articles were published in succession. DNA sequencing for the genome of a bacteriophage was conducted with the Sanger enzymatic dideoxy technique based on chain-terminating dideoxynucleotide analogs.[3,4] A method of DNA sequencing reported in the same year by Maxam and Gilbert and known as Maxam–Gilbert sequencing involves chemical cleavage at specific bases of terminally labeled DNA fragments and separation by gel electrophoresis.[5] The automation of DNA sequence analysis was developed by Caltech (California Institute of Technology, Pasadena, CA, USA) and commercialized by Applied Biosystems (ABI, Applied Biosystems, Foster City, CA, USA), Wilhelm Ansorge at the European Molecular Biology Laboratory and Pharmacia-Amersham, later General Electric Healthcare (GE Healthcare, Little Chalfont, Buckinghamshire, UK).[6–8]

The Sanger method was used in the first automated fluorescent DNA sequencing, in which a complete sequence of 57 kb of the human hypoxanthine-guanine phosphoribosyltransferase (*HGPRT*) gene was determined.[9] ABI introduced the ABI Prism 310 automated DNA sequencer in 1996 and the automated capillary sequencer ABI Prism 3700 in 1998. Together with advances in automation and development of new biochemicals, the Sanger method has made possible the determination of various sequences in many research projects. An initial rough draft of the human genome was finished and announced jointly by US President Bill Clinton and British Prime Minister Tony Blair in 2000, and another study reported the sequencing of the human genome of up to 3 billion bases.[10,11] The first human genome sequence of the Human Genome Project (HGP) was completed in 2003. The HGP has taken 13 years and cost $US 2.7 billion. Using the basic dideoxy method of Sanger sequencing enabled a great achievement.

Before the human genome sequence was completed, Venter proposed a project entitled "The Future of Sequencing: Advancing Towards the $1000 Genome" at the opening session of The Genome Sequencing and

Correspondence: Dr Yoshiyuki Ueno, Department of Gastroenterology, Yamagata University Faculty of Medicine, 2-2-2 Iidanishi Yamagata-shi, Yamagata 990-9585, Japan. Email: y-ueno@med.id.yamagata-u.ac.jp

Received 2 July 2013; revision 2 July 2013; accepted 25 July 2013.

Analysis Conference in 2002 and announced that his foundation would earmark a prize for a breakthrough leading to the goal. Formally, the National Institutes of Health convened the National Human Genome Research Institute and introduced the first in a series of $1000 Genome grants designed to advance the development of breakthrough technologies in 2004. The reaction at the completion of the human genome sequence was different between Japanese and US scientists. While Japanese was considered to have "finished" sequence technology, the USA was thought to have begun. Therefore, some venture companies competed to develop new sequence technology. In 2000, Rothberg established 454 Life Sciences (Branford, CT, USA) and developed the first next-generation sequencing (NGS) system, the GS instrument, in 2005.[12,13] In this review, we describe NGS systems and discuss the application of these advanced technologies in hepatology.

FIRST NGS SYSTEMS: 454 GS SERIES

THE NGS IS now generally defined as the sequencing technology that employs parallel sequencing processes producing thousands or millions of sequence reads simultaneously. Rothberg and colleagues first succeeded in sequencing the *Mycoplasma genitalium* genome with 96% coverage and 99.96% accuracy in a single GS20 run.[13] The GS20 was the first NGS sequencer put on the market by 454 Life Sciences. In the following years, Roche (Basel, Switzerland) absorbed 454 Life Sciences and extended GS20 to a new version of the GS FLX titanium series. The GS FLX titanium series used a parallel pyrosequencing system capable of data output from 100 Mb to 500 Mb per run with a 400–500 bp read length. The pyrosequencing of this sequencer is based on measuring the pyrophosphate generated by the DNA polymerization reaction.[14,15] DNA is fractionated into the fragments of 300–800 bp and these DNA fragments are ligated with short adapters that contain the binding of one fragment to a streptavidin-coated bead. Emulsion polymerase chain reaction (PCR) is carried out for fragment amplification, with water droplets containing one bead and PCR reagents immersed in oil. When the PCR amplification cycles are completed, denaturation beads carrying single-stranded DNA clones are placed into the wells of a fiber-optic slide. On the slide, amplified DNA bound to each of the beads containing sulfurylase and luciferase are sequenced. When one nucleotide is added to the complementary template by the polymerase reaction, a charge-coupled device (CCD) sensor can record the light signal from luciferin. Of note, the signal strength is proportional to the number of nucleotides.[13] This technology is defined as "sequencing-by-synthesis" and is called pyrosequencing in this system.

SECOND-GENERATION NGS SYSTEM: THE ILLUMINA GENOME SEQUENCER

GENERALLY, THE ROCHE/GS FLX titanium series, the Solexa Genome Analyzer (Illumina, San Diego, CA, USA) and the ABI SOLiD system are now classified as second-generation NGS systems. However, the GS FLX series could obtain smaller amounts of data per run than the Illumina or SOLiD systems. Therefore, some technologists believe that Illumina and SOLiD sequencers are second-generation NGS systems.

The Solexa sequencing system, acquired by Illumina, was commercialized in early 2007. The Illumina Genome Analyzer is also based on the "sequencing-by-synthesis" to produce short sequence reads of millions of surface amplified DNA fragments simultaneously. Starting with fragmentation of the genome DNA, adaptor-ligated DNA fragments are attached to the surface of a glass flow cell. The flow cell is separated into eight channels and the surfaces of the channels have covalently attached oligos complementary to the adaptors and ligated to the library DNA fragments. Then, on the flow cell, adaptor-ligated DNA fragments are amplified by polymerase reaction for bridge amplification and form the cluster fragments. The cluster fragments are denatured, annealed with a sequencing primer and subjected to DNA synthesis with four differentially reversible labeled fluorescent nucleotides that have their 3′-end chemical termination to ensure that only a single base is extended. After a single base is incorporated into the DNA strand, the terminator nucleotide is detected via its labeled fluorescent dye by the CCD camera. Then, the labeled fluorescent dye and 3′-end chemical terminations are removed and the next DNA synthesis cycle is repeated.

The Genome Analyzer IIx can obtain 30–100 nucleotide read lengths and data output per paired-end run from 1–3 Gb.[16–18] Moreover, Illumina released a HiSeq sequencer series which enabled higher throughput and a desktop MiSeq sequencer type which could sequence more rapidly in 2013.

SECOND-GENERATION NGS SYSTEM: THE ABI SOLiD SEQUENCER

THE ABI SOLID sequencer was introduced in October 2007. SOLiD is an abbreviation for "sequencing

by oligo ligation and detection". It uses a unique sequencing method catalyzed by DNA ligase. The universal P1 adaptor-linked DNA fragments are attached to magnetic beads. Emulsion PCR is conducted in microreactors containing the reagents of the PCR reaction. The magnetic beads are covalently attached to the surface of a specially treated glass slide that is placed into a fluidic cassette within the sequencer. The universal sequence primers hybridize to the P1 adapter within the library template. The set of four fluorescent-labeled di-base 8-mer probes are annealed to the sequencing primer and library template. Identification of the nucleotide sequence by the 8-mer probe is achieved by interrogating every first and second base in each ligation reaction. When there is a matching of the 8-mer probe to the library template adjacent to the universal primer of the 3'-end, DNA ligase seals the phosphate backbone. After the ligation, the probe is enzymatically removed together with the last three bases attaching the linkage between base 5 and 6. Then, the same probe hybridizing process is conducted and the sequence data of each library template can be obtained at five nucleotide intervals. Following a series of ligation cycles, the library template is reset with five rounds of universal primers complementary to the n to n-4 position for a multistep round of ligation cycles. Through the primer reset process, each base is interrogated in two independent ligation reactions by two different primers and the nucleotide sequence is defined by this repetition.

The ABI SOLiD 2.0 platform, produced in 2008, can obtain data output from 3–10 Gb per run.[16–18]

THIRD- AND FOURTH-GENERATION NGS SYSTEM

HOWEVER, MUCH IMPROVED the NGS systems have already become, the competition in technology development is intensifying. The demand for low cost, high speed and highly accurate systems has spurred development beyond third-generation NGS systems (Table 1).

Ion semiconductor sequencing was introduced by Ion Torrent Systems (South San Francisco, CA, USA) and was released in 2010. The principle of the technology is based on detecting hydrogen ions released in the reaction-induced changes of the pH of the solution by an ion sensor when the nucleotide base is incorporated by DNA polymerase. Its read length of approximately 100 bp is comparable to that of other NGS systems, but the throughput is still lower, although increasing the size of the semiconductor chips could improve the throughput.[19,20]

Pacific Biosciences (PacBio, Menlo Park, CA, USA) developed a single molecule real-time sequencer based on single molecule real-time sequencing by the synthesis method with monitoring of the deoxyribonucleotide triphosphate (dNTP) uptake of DNA sequencing by DNA polymerase. The fluorescently labeled dNTP is incorporated and the fluorescent dye is separated from the DNA. The sequencing reaction is conducted on zero-mode wave guides (ZMW) that are small well-containers with detectors located at the bottom of the well. The detectors can capture the fluorescent dye. The

Table 1 The classification of NGS systems

Classification	Second-generation	Third-generation	Fourth-generation
Principal	Using "sequencing-by-synthesis" method by DNA polymerase or DNA ligase, the nucleotide sequence is determined in parallel by optical detection such as fluorescence emission	By carrying out the DNA synthesis by DNA polymerase referring as one template DNA molecule, the nucleotide sequence is determined in real time by detecting fluorescence emission with one base sequencing reaction	The nucleotide sequence is determined by nanopore sequencing or by a method without the detection of fluorescent or luminescence emission
Throughput	1–50 Gy/day	45–60 Mb/run	>100 Mb/run (Ion Torrent)
Accuracy	Excellent	Error rate is relatively large	Excellent
Read length	25–700 bases	1–2 kilobases	100–200 bases (Ion Torrent)
Sequencer	Roche454 Illumina sequencers ABI SOLiD	PacBio Genia	Ion Torrent systems Oxford Nanopore

Table 2 Classification of next-generation sequencing (NGS) system by application

	Application	NGS system	Features
Research use	Advanced research application	PacBio RS Oxford Nanopore GridION	Single-molecule real-time sequencing, detection of DNA methylation and long-read sequencing
	General genome research application	Illumina HiSeq/Genome Analyzer IIx ABI SOLiD Roche 454 GS Ion Torrent Proton	Whole-genome sequencing with high throughput. Advanced knowledge of molecular biology is necessary for sequencing analysis
Clinical use	Clinical diagnosis application	Illumina MiSeq Ion Torrent PGM Oxford Nanopore MinION	Desktop type or USB memory stick type. Automatic extraction of DNA from samples or sequencing analysis

DNA polymerase is immobilized by only one molecule at the bottom. After the single template DNA is bound to the polymerase with incorporation of the fluorescently labeled dNTP, the DNA synthesis is performed. The DNA sequencing is conducted by detecting the separated fluorescent dye.[21] In January 2011, a paper from PacBio was published in the *New England Journal of Medicine* demonstrating the origin of the 2010 cholera outbreak in Haiti.[22] The PacBio RS was commercially released in early 2011 and had the advantage of a short time from equipping the library to sequencing, obtaining long reads and fewer errors or bias with PCR amplification. However, there is the disadvantage of low yield at high accuracy and low throughput.

Nanopore sequencing technology has been developing since 1995 for determining the sequence without nucleotide labeling and detection.[23] In brief, DNA sequencing with nanopore technology relies on the conversion of the electrical signals of nucleotides by passing through a nanopore, which is a specific protein pore covalently attached to the molecules. This approach is the most advanced and was demonstrated by Oxford Nanopore Technologies (Oxford Science Park, Oxford, UK).[24] Two nanopore sequencer models, the GridION sequencer which can perform large-scale sequencing, and the MinION sequencer, which is a portable and disposable sequencer, are planned for release. The MinION sequencer is a breakthrough device that overturns the concept of previous sequencers. The size of this sequencer is almost the same as a Universal Serial Bus (USB) memory stick and, after plugging this sequencer into the USB port of a personal computer, sequencing can be performed just by loading the sample. So far, this nanopore sequencer has tremendously surpassed other NGS systems. But there is a problem in that the error rate is still high compared with the Illumina or SOLiD sequencers.

Genia Technologies is a venture company founded in Mountain View city (CA, USA) in March 2009. They are now planning to launch the Genia sequencer in 2013. Genia technology combines the complementary metal–oxide–semiconductor (CMOS) chip technology of Ion Torrent and the nanopore sequencing by Stefan Roever.

The race to develop NGS systems is being carried out with the goal of "lower cost and higher performance". Therefore, we cannot select a sequencer in any appropriate analysis. We classified the three types of NGS systems for different applications. Type 1 (advanced research application) includes sequencers such as the PacBio RS or Oxford nanopore GridION, which can detect DNA methylation and perform long-read sequencing. Type 2 (general genome research application) includes sequencers such as the Illumina sequencer series or ABI SOLiD or Ion Torrent sequencers, which can be used for whole-genome sequencing with high throughput. Advanced knowledge of molecular biology is necessary for sequencing analysis. Type 3 (clinical diagnosis application) includes the Nanopore MinION, which can automatically conduct the extraction DNA from samples and the sequencing analysis (Table 2).

NGS APPLICATIONS TO VIRAL HEPATITIS

SINCE THE INTRODUCTION of the NGS sequencer in 2005, the production of large numbers of sequence reads made useful for many applications concerned with human genomes research, particularly whole-genome resequencing, de novo genome sequencing or transcriptomes (RNA-seq), genomic variation and mutation detection, genome-wide profiling of epigenetic marks and chromatin structure using ChIP-seq.

Currently, the identification of viral genome sequences is mainly cloning by PCR amplification with Sanger direct sequencing. Usually, viruses infecting a host have genomic diversity, referred to as "quasispecies". However, with this method it is difficult to measure the frequencies of each mutation, and it is impossible to detect several mutations combined in the same sequence. As an alternative to Sanger direct sequencing, molecular cloning can analyze single viral DNA molecules. However, this methodology is complicated and time-consuming. These complications can now be overcome by NGS technology. Therefore, this technology is suitable for whole viral genome sequencing, metagenomics, the identification of viral variants and viral dynamics. Some of the topics related to the clinical application for hepatitis virus will be described.

Hepatitis C virus (HCV)

The appearance of HCV variants is generated because of the high replication rate and the error-prone nature of RNA-dependent RNA polymerase. The selection of the mutants has developed to escape immunological and therapeutic control.[25] Moreover, the presence of contaminating nucleic acids of the host cell and other viral agents make it difficult to sequence the full-length HCV genome. In fact, the preparation of a library of cDNA synthesized from RNA with random priming results in a huge amount of host-specific genomes instead of the viral sequences, even in the presence of a very high viral load. High throughput sequencing techniques could be used to obtain sufficient sequence coverage, but the lengths of reads might be too short to allow de novo assembly, and the method of mapping to the reference HCV genome could be detecting the full-length HCV sequence. NGS technology is a powerful tool for obtaining more profound insight into the dynamics of genetic variants in the HCV quasispecies in human serum.[26]

Currently, potential treatments in development include drugs that target the HCV NS3/4A serine protease and the NS5B RNA-dependent RNA polymerase referred to as direct-acting antiviral agent (DAA).[27] These drugs have been evaluated in clinical trials alone and in combination with pegylated interferon and ribavirin.[28] Therefore, detecting the frequency of drug-resistant HCV variants prior to treatment is important. In treatment-naïve patients, the frequency of all resistant variants of NS3 was generally found to be below 1% using the 454 GS series[29,30] or by the Illumina Genome sequencer.[31] Viral dynamics have emerged whereby drug-resistant variants frequently appear, but are rapidly lost in the absence of selective pressure because of reduced fitness. Results using NGS technology have also suggested that not only the number but also the nature of the nucleotide changes can contribute to the genetic barriers to the development of resistance to DAAs.[32] Using a genetically engineered HCV infection system in a chimeric mouse model, the rapid emergence of DAA-resistant HCV variants was induced by mutation from a wild-type strain of HCV *in vivo*.[33]

Other 454 GS series sequencer studies showed that analysis of the PKR-elf2α phosphorylation homology domain sequence before or during treatment cannot be used to reliably predict the outcome of treatment in patients co-infected with HCV genotype 1 and HIV,[34] and highlighted the genetic diversity of HCV, which enables it to evade the host immune system.[35] Concerning the within-host evolution of HCV during infection, the genetic diversity of viral variants showed strong selective forces that limit viral evolution in the acute phase of infection.[36,37]

Hepatitis B virus (HBV)

Taking nucleoside/nucleotide analogs (NA) is a major antiviral therapy for the treatment of chronic HBV infection.[38] They inhibit the viral polymerase activity by interfering with the priming of reverse transcription and elongation of the viral minus or plus strand DNA.[39] The problem is that these treatments are hampered by the selection of drug-resistant mutants, leading to a loss of efficacy, viral relapse and exacerbations of hepatitis after discontinuation.[40]

Using NGS, drug-resistant mutations of HBV minor variants can be identified. The dynamics of emerging NA-resistant HBV variants are not well understood because standard Sanger sequencing methods detect drug-resistance mutations only after they have become dominant. NGS methods may offer significant advantages in explaining and predicting the responses of patients with HBV to antiviral therapy. In the sequential analysis of the region encoded reverse transcriptase, NA-resistant HBV variants were present in combinations of amino acid substitutions that increased in complexity after viral breakthrough or unsuccessful therapy with NA, at which time the combined NA-resistant variants predominated and the pretreatment HBV variants did not show NA-resistant motifs.[41–43] In another study, primarily NA resistance-related mutant variants were found to exist with minor variants in treatment-naïve patients.[44]

Hepatocellular carcinoma (HCC)

Despite its global importance, HCC is understudied compared to other major lethal cancers and we have a

little knowledge of the genomic alterations related to the initiation and progression of HCC. This may be due to the high complexity of the HCC cancer genome, which simple genomic approaches cannot easily simplify. Previous studies have revealed several genetic aberrations in HCC, including point mutations in p53[45] and Wnt-activating β-catenin,[46] hepatocyte-specific Pten deficiency,[47] the interaction of c-Myc and transforming growth factor (TGF)-α,[48] overexpression of the proto-oncogene MET[49] or cyclin D1/TGF-β1,[50] and HBV integrations into the TERT and MLL4 gene loci that encode telomerase reverse transcriptase and histone lysine methyl transferase, respectively. The gene expression profiles of HCC have been gradually revealed and suggest the therapeutic potential for genetic targets.[51] However, knowledge of the genetic background in HCC is far from complete and the molecular changes of HCC tumorigenesis remain poorly understood. We have summarized the HCC information concerning related genes discovered by NGS technology from Europe and Asia. In 2012, Fujimoto *et al.* detected that multiple chromatin regulators, including *ARID1A*, *ARID1B*, *ARID2*, *MLL* and *MLL3*, were mutated in approximately 50% of the HCC and the HBV genome was frequently integrated in the *TERT* locus, as determined by whole-genome sequencing analysis by Illumina NGS sequencers.[52] A European group also found new recurrent alternations of *ARID1A*, *RPS6KA3*, *NFE2L2* and *IRF2*. In addition, Wnt/β-catenin signaling, related to the mutations of *RPS6KA3-AXIN1* and *NFE2L2-CTNNB1*, may be involved in liver carcinogenesis together with both oxidative stress metabolism and Ras/mitogen-activated protein kinase (MAPK).[53]

CONCLUSION

GIVEN THE RAPID development of NGS systems, the goal of determining a whole-genome sequence for $US 1000 could become feasible in the near future. The cost of sequencing has become greatly reduced, and "one cell" or "one molecule" sequencing has become possible. By performing whole-genome sequencing, RNA sequencing and epigenetic analysis at one cellular level, dynamic genomic changes can be followed with time-course analysis in the same cells or for differences between various cells. When analyzing the genome sequence by removing the cancer tissue, the data of a mixture of cancer cells and normal cells can be usually obtained. Cancer cells are usually changed at the genomic level; therefore, mixture sequence data of multiple species can be obtained in some cases. If the genome sequence of the cancer tissue can be determined at a one-cell level, we will obtain a more accurate understanding of the progress and development of the cancer. Moreover, with the development of the NGS systems, analysis of DNA and RNA sequencing at the intracellular level proceed. Single molecule sequencing of cDNA converted to mRNA by the Nanopore sequencer can accurately represent the structure of the mixed mRNA containing splicing variants and clarify their intracellular distribution. Using short-read NGS, a large number of sequence reads are obtained making it possible to analyze variants or mutants in the virus population. The application of this novel technique includes the profiling of disease-specific gene expressions. Recently, we have successfully demonstrated that serum samples from patients with primary biliary cirrhosis had a distinct miRNA expression profile using NGS.[54] As such technologies develop further, new applications can also be expected to appear.

REFERENCES

1 Schuster SC. Next-generation sequencing transforms today's biology. *Nat Methods* 2007; **5**: 16–8.
2 Sanger F, Coulson AR. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *J Mol Biol* 1975; **94**: 441–8.
3 Sanger F, Air GM, Barrell BG *et al.* Nucleotide sequence of bacteriophage phi X174 DNA. *Nature* 1977; **265**: 687–95.
4 Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* 1977; **74**: 5463–7.
5 Maxam AM, Gilbert W. A new method for sequencing DNA. *Proc Natl Acad Sci U S A* 1977; **74**: 560–4.
6 Smith LM, Sanders JZ, Kaiser RJ *et al.* Fluorescence detection in automated DNA sequence analysis. *Nature* 1986; **321**: 674–9.
7 Ansorge W, Sproat BS, Stegemann J, Schwager C. A non-radioactive automated method for DNA sequence determination. *J Biochem Biophys Methods* 1986; **13**: 315–23.
8 Ansorge W, Sproat B, Stegemann J, Schwager C, Zenke M. Automated DNA sequencing: ultrasensitive detection of fluorescent bands during electrophoresis. *Nucleic Acids Res* 1987; **15**: 4593–602.
9 Edwards A, Voss H, Rice P *et al.* Automated DNA sequencing of the human HPRT locus. *Genomics* 1990; **6**: 593–608.
10 Lander ES, Linton LM, Birren B *et al.* Initial sequencing and analysis of the human genome. *Nature* 2001; **409**: 860–921.
11 Venter JC, Adams MD, Myers EW *et al.* The sequence of the human genome. *Science* 2001; **291**: 1304–51.
12 Pareek CS, Smoczynski R, Tretyn A. Sequencing technologies and genome sequencing. *J Appl Genet* 2011; **52**: 413–35.

13 Margulies M, Egholm M, Altman WE *et al.* Genome sequencing in microfabricated high-density picolitre reactors. *Nature* 2005; 437: 376–80.
14 Nyren P, Lundin A. Enzymatic method for continuous monitoring of inorganic pyrophosphate synthesis. *Anal Biochem* 1985; 151: 504–9.
15 Hyman ED. A new method of sequencing DNA. *Anal Biochem* 1988; 174: 423–36.
16 Ansorge WJ. Next-generation DNA sequencing techniques. *N Biotechnol* 2009; 25: 195–203.
17 Mardis ER. The impact of next-generation sequencing technology on genetics. *Trends Genet* 2008; 24: 133–41.
18 Mardis ER. Next-generation DNA sequencing methods. *Annu Rev Genomics Hum Genet* 2008; 9: 387–402.
19 Pennisi E. Genomics. Semiconductors inspire new sequencing technologies. *Science* 2010; 327: 1190.
20 Rusk N. Torrents of sequence. *Nat Methods* 2011; 8: 44.
21 Levene MJ, Korlach J, Turner SW, Foquet M, Craighead HG, Webb WW. Zero-mode waveguides for single-molecule analysis at high concentrations. *Science* 2003; 299: 682–6.
22 Chin CS, Sorenson J, Harris JB *et al.* The origin of the Haitian cholera outbreak strain. *N Engl J Med* 2011; 364: 33–42.
23 Kasianowicz JJ, Brandin E, Branton D, Deamer DW. Characterization of individual polynucleotide molecules using a membrane channel. *Proc Natl Acad Sci U S A* 1996; 93: 13770–3.
24 Clarke J, Wu HC, Jayasinghe L, Patel A, Reid S, Bayley H. Continuous base identification for single-molecule nanopore DNA sequencing. *Nat Nanotechnol* 2009; 4: 265–70.
25 Rong L, Dahari H, Ribeiro RM, Perelson AS. Rapid emergence of protease inhibitor resistance in hepatitis C virus. *Sci Transl Med* 2010; 2: 30ra32.
26 Ninomiya M, Ueno Y, Funayama R *et al.* Use of illumina deep sequencing technology to differentiate hepatitis C virus variants. *J Clin Microbiol* 2011; 50: 857–66.
27 De Francesco R, Migliaccio G. Challenges and successes in developing new therapies for hepatitis C. *Nature* 2005; 436: 953–60.
28 Pawlotsky JM, Chevaliez S, McHutchison JG. The hepatitis C virus life cycle as a target for new antiviral therapies. *Gastroenterology* 2007; 132: 1979–98.
29 Fonseca-Coronado S, Escobar-Gutierrez A, Ruiz-Tovar K *et al.* Specific detection of naturally occurring hepatitis C virus mutants with resistance to telaprevir and boceprevir (protease inhibitors) among treatment-naive infected individuals. *J Clin Microbiol* 2012; 50: 281–7.
30 Lauck M, Alvarado-Mora MV, Becker EA *et al.* Analysis of hepatitis C virus intrahost diversity across the coding region by ultradeep pyrosequencing. *J Virol* 2012; 86: 3952–60.
31 Nasu A, Marusawa H, Ueda Y *et al.* Genetic heterogeneity of hepatitis C virus in association with antiviral therapy determined by ultra-deep sequencing. *PLoS ONE* 2011; 6: e24907.
32 Powdrill MH, Tchesnokov EP, Kozak RA *et al.* Contribution of a mutational bias in hepatitis C virus replication to the genetic barrier in the development of drug resistance. *Proc Natl Acad Sci U S A* 2011; 108: 20509–13.
33 Hiraga N, Imamura M, Abe H *et al.* Rapid emergence of telaprevir resistant hepatitis C virus strain from wildtype clone in vivo. *Hepatology* 2011; 54: 781–8.
34 Bolcic F, Sede M, Moretti F *et al.* Analysis of the PKR-eIF2alpha phosphorylation homology domain (PePHD) of hepatitis C virus genotype 1 in HIV-coinfected patients by ultra-deep pyrosequencing and its relationship to responses to pegylated interferon-ribavirin treatment. *Arch Virol* 2012; 157: 703–11.
35 Merani S, Petrovic D, James I *et al.* Effect of immune pressure on hepatitis C virus evolution: insights from a single-source outbreak. *Hepatology* 2011; 53: 396–405.
36 Bull RA, Luciani F, McElroy K *et al.* Sequential bottlenecks drive viral evolution in early acute hepatitis C virus infection. *PLoS Pathog* 2011; 7: e1002243.
37 Wang GP, Sherrill-Mix SA, Chang KM, Quince C, Bushman FD. Hepatitis C virus transmission bottlenecks analyzed by deep sequencing. *J Virol* 2010; 84: 6218–28.
38 Lok ASF, McMahon BJ. Chronic hepatitis B: update 2009. *Hepatology* 2009; 50: 661–2.
39 Seifer M, Hamatake RK, Colonno RJ, Standring DN. In vitro inhibition of hepadnavirus polymerases by the triphosphates of BMS-200475 and lobucavir. *Antimicrob Agents Chemother* 1998; 42: 3200–8.
40 Inada M, Yokosuka O. Current antiviral therapies for chronic hepatitis B. *Hepatol Res* 2008; 38: 535–42.
41 Tavis JE, Rodriguez-Frías F, Tabernero D *et al.* Ultra-deep pyrosequencing detects conserved genomic sites and quantifies linkage of drug-resistant amino acid changes in the hepatitis B virus genome. *PLoS ONE* 2012; 7: e37874.
42 Sede M, Ojeda D, Cassino L *et al.* Long-term monitoring drug resistance by ultra-deep pyrosequencing in a chronic hepatitis B virus (HBV)-infected patient exposed to several unsuccessful therapy schemes. *Antiviral Res* 2012; 94: 184–7.
43 Margeridon-Thermet S, Shulman NS, Ahmed A *et al.* Ultra-deep pyrosequencing of hepatitis B virus quasispecies from nucleoside and nucleotide reverse-transcriptase inhibitor (NRTI)-treated patients and NRTI-naive patients. *J Infect Dis* 2009; 199: 1275–85.
44 Han Y, Huang LH, Liu CM *et al.* Characterization of hepatitis B virus reverse transcriptase sequences in Chinese treatment naive patients. *J Gastroenterol Hepatol* 2009; 24: 1417–23.
45 Klocke R, Bartels T, Jennings G *et al.* Lack of p53 accelerates hepatocarcinogenesis in transgenic mice constitutively overexpressing c-myc in the liver. *FASEB J* 2001; 15: 1404–6.

46 Harada N, Oshima H, Katoh M, Tamai Y, Oshima M, Taketo MM. Hepatocarcinogenesis in mice with beta-catenin and Ha-ras gene mutations. *Cancer Res* 2004; 64: 48–54.
47 Horie Y, Suzuki A, Kataoka E *et al.* Hepatocyte-specific Pten deficiency results in steatohepatitis and hepatocellular carcinomas. *J Clin Invest* 2004; **113**: 1774–83.
48 Murakami H, Sanderson ND, Nagy P, Marino PA, Merlino G, Thorgeirsson SS. Transgenic mouse model for synergistic effects of nuclear oncogenes and growth factors in tumorigenesis: interaction of c-myc and transforming growth factor alpha in hepatic oncogenesis. *Cancer Res* 1993; **53**: 1719–23.
49 Tward AD, Jones KD, Yant S, Kay MA, Wang R, Bishop JM. Genomic progression in mouse models for liver tumors. *Cold Spring Harb Symp Quant Biol* 2005; **70**: 217–24.
50 Deane NG, Lee H, Hamaamen J *et al.* Enhanced tumor formation in cyclin D1 x transforming growth factor beta1 double transgenic mice with characterization by magnetic resonance imaging. *Cancer Res* 2004; 64: 1315–22.
51 Lee J-S, Chu I-S, Heo J *et al.* Classification and prediction of survival in hepatocellular carcinoma by gene expression profiling. *Hepatology* 2004; **40**: 667–76.
52 Fujimoto A, Totoki Y, Abe T *et al.* Whole-genome sequencing of liver cancers identifies etiological influences on mutation patterns and recurrent mutations in chromatin regulators. *Nat Genet* 2012; 44: 760–4.
53 Guichard C, Amaddeo G, Imbeaud S *et al.* Integrated analysis of somatic mutations and focal copy-number changes identifies key genes and pathways in hepatocellular carcinoma. *Nat Genet* 2012; 44: 694–8.
54 Ninomiya M, Kondo Y, Funayama R *et al.* Distinct microRNAs expression profile in primary biliary cirrhosis and evaluation of miR 505-3p and miR197-3p as novel biomarkers. *PLoS ONE* 2013; **8**: e66086.

JOURNAL OF VIRAL HEPATITIS

Journal of Viral Hepatitis, 2014, 21, 762–768 doi:10.1111/jvh.12312

Sofosbuvir plus ribavirin in Japanese patients with chronic genotype 2 HCV infection: an open-label, phase 3 trial

Masao Omata,[1] Shuhei Nishiguchi,[2] Yoshiyuki Ueno,[3] Hitoshi Mochizuki,[1] Namiki Izumi,[4] Fusao Ikeda,[5] Hidenori Toyoda,[6] Osamu Yokosuka,[7] Kazushige Nirei,[8] Takuya Genda,[9] Takeji Umemura,[10] Tetsuo Takehara,[11] Naoya Sakamoto,[12] Yoichi Nishigaki,[13] Kunio Nakane,[14] Nobuo Toda,[15] Tatsuya Ide,[16] Mikio Yanase,[17] Keisuke Hino,[18] Bing Gao,[19] Kimberly L. Garrison,[19] Hadas Dvory-Sobol,[19] Akinobu Ishizaki,[19] Masa Omote,[19] Diana Brainard,[19] Steven Knox,[19] William T. Symonds,[19] John G. McHutchison,[19] Hiroshi Yatsuhashi[20] and Masashi Mizokami[17]

[1]*Yamanashi Prefectural Hospital Organization, Yamanashi, Japan;* [2]*Hyogo College of Medicine, Hyogo, Japan;* [3]*Yamagata University, Yamagata, Japan;* [4]*Musashino Red Cross Hospital, Tokyo, Japan;* [5]*Okayama University, Okayama, Japan;* [6]*Ogaki Municipal Hospital, Gifu, Japan;* [7]*Chiba University, Chiba, Japan;* [8]*Nihon University, Tokyo, Japan;* [9]*Juntendo University, Tokyo, Japan;* [10]*Shinshu University, Nagano, Japan;* [11]*Osaka University, Osaka, Japan;* [12]*Hokkaido University, Hokkaido, Japan;* [13]*Gifu Municipal Hospital, Gifu, Japan;* [14]*Akita City Hospital, Akita, Japan;* [15]*Mitsui Memorial Hospital, Tokyo, Japan;* [16]*Kurume University, Kurume, Japan;* [17]*National Center for Global Health and Medicine, Tokyo, Japan;* [18]*Kawasaki Medical School, Okayama, Japan;* [19]*Gilead Sciences, Inc., Foster City, CA, USA; and* [20]*National Hospital Organization Nagasaki Medical Center, Nagasaki, Japan*

Received June 2014; accepted for publication August 2014

SUMMARY. Genotype 2 hepatitis C virus (HCV) accounts for up to 30% of chronic HCV infections in Japan. The standard of care for patients with genotype 2 HCV – peginterferon and ribavirin for 24 weeks – is poorly tolerated, especially among older patients and those with advanced liver disease. We conducted a phase 3, open-label study to assess the efficacy and safety of an all-oral combination of the NS5B polymerase inhibitor sofosbuvir and ribavirin in patients with chronic genotype 2 HCV infection in Japan. We enrolled 90 treatment-naïve and 63 previously treated patients at 20 sites in Japan. All patients received sofosbuvir 400 mg plus ribavirin (weight-based dosing) for 12 weeks. The primary endpoint was sustained virologic response at 12 weeks after therapy (SVR12). Of the 153 patients enrolled and treated, 60% had HCV genotype 2a, 11% had cirrhosis, and 22% were over the aged 65 or older. Overall, 148 patients (97%) achieved SVR12. Of the 90 treatment-naïve patients, 88 (98%) achieved SVR12, and of the 63 previously treated patients, 60 (95%) achieved SVR12. The rate of SVR12 was 94% in patients with cirrhosis and in those aged 65 and older. No patients discontinued study treatment due to adverse events. The most common adverse events were nasopharyngitis, anaemia and headache. Twelve weeks of sofosbuvir and ribavirin resulted in high rates of SVR12 in treatment-naïve and previously treated patients with chronic genotype 2 HCV infection. The treatment was safe and well tolerated by patients, including the elderly and those with cirrhosis.

Keywords: Hepatitis C virus, HCV genotype 2, direct-acting antiviral agents, nucleotide polymerase inhibitor.

INTRODUCTION

Approximately two million people in Japan – nearly 2% of the population – are chronically infected with the hepatitis C virus (HCV) [1]. The population of patients with chronic HCV infection in Japan differs from that of other countries; patients are generally older, have more advanced liver disease and are more likely to have received previous treatment for HCV infection [2,3]. It is estimated that 15–30% of Japanese patients with HCV will develop serious complications, including liver cirrhosis, end-stage liver disease and hepatocellular carcinoma [4]. Although genotype 1 HCV is currently the most prevalent strain of the virus in Japan, genotype 2 HCV, which now accounts for up to 30% of infections, is rising in prevalence [5]. The current standard of care regimen for the treatment of chronic genotype 2 HCV infection in Japan is 24 weeks of pegylated interferon alpha (Peg-IFNα) and ribavirin (RBV) [6]. Although relatively high rates of SVR

Abbreviations: CI, confidence interval; GCP, Good Clinical Practice; HCV, hepatitis C virus; ICH, International Conference on Harmonization; Peg-IFNα, pegylated interferon alpha; PK, pharmacokinetics; RBV, ribavirin; SVR12, 12 weeks after therapy.

Correspondence: Masao Omata, Yamanashi Prefectural Hospital Organization, 1-1-1 Fujimi, Kofu City, Yamanashi 400-0027, Japan. E-mail: momata-tky@umin.ac.jp

Trial registration details: ClinicalTrials.gov number NCT01910636.