With this background in mind, the present study was initiated to investigate the cumulative incidence and risk factors of malignancies, including HCC after prolonged follow-up in HCV patients treated with interferon (IFN) monotherapy or combination therapy of IFN and ribavirin. The strengths of the current study are the large numbers of patients included and the long-term follow-up of patients.

Patients and Methods

Patients. The number of patients who were diagnosed with chronic HCV infection and treated for the first time with IFN monotherapy or combination therapy between September 1990 and March 2009 in the Department of Hepatology, Toranomon Hospital, Tokyo, Japan, was 7,205. Of these, 4,302 patients met the following enrollment criteria: (1) no evidence of malignancies by physical examination, biochemical tests, abdominal ultrasonography, gastrofiberscope (or gastrography), or chest X-ray (or computed tomography); (2) features of chronic hepatitis or cirrhosis diagnosed via laparoscopy and/or liver biopsy within 1 year before the initiation of IFN therapy; (3) positivity for serum HCV-RNA before the initiation of IFN therapy; (4) period of $\geq$1 month to $\leq$1 year of IFN therapy; (5) negativity for hepatitis B surface antigens, antibody to hepatitis B core, or antimitochondrial antibodies in serum, as determined by radioimmunoassay, enzyme-linked immunosorbent assay, or indirect immunofluorescence assay; (6) age of $\geq$30 years to $\leq$80 years; (7) no underlying systemic disease, such as systemic lupus erythmatosus or rheumatic arthritis; and (8) repeated annual examinations during follow-up. Annual examinations included biochemical tests, tumor marker (carcinoembryonic antigen, alpha-fetoprotein, and prostate-specific antigen [only in men]), and abdominal ultrasonography. Patients with were excluded from the study if they had illnesses that could seriously reduce their life expectancy or if they had a history of carcinogenesis.

The primary outcome was the first development of malignancy. The development of malignancies was diagnosed by clinical symptoms, tumor marker, imaging (ultrasonography, computed tomography, or magnetic resonance imaging), and/or histological examination.[9-15] All of the studies were performed retrospectively by collecting and analyzing data from the patient records. The physicians in charge explained the purpose, method, and side effects of IFN therapy to each patient and/or the patient's family. In addition, the physicians in charge received permission for the use of serum stores and future use of stored serum. Informed consent for IFN therapy and future use of stored serum was obtained from all patients. The study was approved by the Institutional Review Board of our hospital.

Medical Evaluation. Body weight was measured in light clothing and without shoes to the nearest 0.1 kg. Height was measured to the nearest 0.1 cm. Height and weight were recorded at baseline, and body mass index was calculated as kg/m^2. All patients were interviewed by physicians or nurse staff in the Toranomon Hospital using a questionnaire that gathered information on demographic characteristics, medical history, and heath-related habits, including questions on alcohol intake and smoking history.

The value for hemoglobin A_{1C} (HbA_{1C}) was estimated as a National Glycohemoglobin Standardization Program equivalent value (%). Patients were defined as having T2DM when they had a fasting plasma glucose level of $\geq$126 mg/dL and/or HbA_{1C} level of $\geq$6.5%.[16]

Patients were regarded as hypertensive when systolic blood pressure was $\geq$140 mm Hg and/or diastolic blood pressure was $\geq$90 mm Hg for at least three visits. Smoking index (packs per day $\times$ year) and total alcohol intake (TAI) were evaluated by the sum of before, during, and after the IFN therapy.

Laboratory Investigation. Diagnosis of HCV infection was based on detection of serum HCV antibody and positive RNA. Anti-HCV was detected using an enzyme-linked immunosorbent assay (ELISA II; Abbott Laboratories, North Chicago, IL). HCV genotype was examined via polymerase chain reaction assay, using a mixture of primers for the six subtypes known to exist in Japan, as reported.[17] HCV-RNA was determined using the COBAS TaqMan HCV test (Roche Diagnostics, Basel, Switzerland). The serum samples stored at -80°C before IFN therapy were used. The linear dynamic range of the assay was 1.2-7.8 log IU/

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Potential conflict of interest: Dr. Suzuki is on the speakers' bureau of Bristol-Myers Squibb. Dr. Akuta is on the speakers' bureau of MSD and holds intellectual property rights with SRL. Dr. Kumada is on the speakers' bureau of MSD, Mitsubishi Tanabe Pharma, Dainippon Sumitomo Pharma, Bristol-Myers Squibb. He also holds intellectual property rights with SRL. Dr. Arase is on the speakers' bureau of MSD.

Table 1. Clinical Backgrounds at Initiation of Follow-up in Enrolled Patients

Variable	Total	HCC Group	Non-HCC Malignancy Group	Without Events Group	P
No. of patients	4,302	393	213	3,696	
Age, years	52.0 ± 11.8	55.8 ± 7.9	57.9 ± 9.1	51.3 ± 12.1	<0.001
Sex, male/female	2528/1774	272/121	129/84	2127/1569	<0.001
Height, cm	163.0 ± 9.2	162.8 ± 8.3	163.3 ± 9.1	163.0 ± 9.3	0.772
Weight, kg	61.4 ± 13.0	62.3 ± 10.6	60.8 ± 10.1	61.3 ± 13.4	0.142
BMI	23.0 ± 4.0	23.4 ± 3.0	22.8 ± 2.8	23.0 ± 4.1	0.012
Blood pressure, mm Hg					
Systolic	128 ± 18	132 ± 19	133 ± 20	127 ± 17	<0.001
Diastolic	77 ± 13	80 ± 12	80 ± 13	77 ± 13	<0.001
TAI, kg*	95 ± 92	151 ± 101	135 ± 81	85 ± 89	<0.001
Smoking index*	6.4 ± 9.4	10.8 ± 11.1	12.5 ± 11.8	5.5 ± 8.7	<0.001
AST, IU/L	42 ± 44	64 ± 55	42 ± 31	40 ± 42	<0.001
ALT, IU/L	44 ± 53	72 ± 63	43 ± 43	42 ± 52	<0.001
GGT, IU/L	54 ± 61	63 ± 65	56 ± 45	53 ± 38	0.007
Albumin, g/dL	4.1 ± 0.3	4.1 ± 0.3	4.1 ± 0.2	4.1 ± 0.2	0.310
Triglyceride, mg/dL	101 ± 53	104 ± 54	105 ± 50	100 ± 52	0.329
Cholesterol, mg/dL	170 ± 32	165 ± 31	169 ± 33	171 ± 32	0.025
FPG, mg/dL	100 ± 22	110 ± 26	104 ± 22	98 ± 21	<0.001
HbA1c, %, NSPG	5.6 ± 1.2	5.9 ± 1.4	5.7 ± 1.4	5.5 ± 1.1	<0.001
T2DM, +/−	267/4,035	63/330	34/179	170/3,526	<0.001
Platelet count, $\times 10^4/mm^3$	17.1 ± 5.1	13.7 ± 4.9	16.5 ± 5.4	17.5 ± 5.4	<0.001
Staging, LC/non-LC	433/3,869	113/285	27/189	293/3,395	<0.001
HCV genotype, 1b/2a/2b/other	2,721/995/458 /128	283/52/20/38	121/62/18/12	2,317/881/420/78	<0.001
HCV RNA, log IU/mL	6.06 ± 1.05	6.22 ± 0.52	6.05 ± 0.86	6.04 ± 1.05	0.003
IFN monotherapy†/combination therapy‡	2,861/1,441	358/35	175/38	2,328/1,368	<0.001
Efficacy, SVR/non-SVR	1,900/2,402	44/349	88/125	1,768/1,928	<0.001

Data are presented as no. of patients or mean ± SD.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; F, female; FPG, fasting plasma glucose; GGT, gamma-glutamyl transferase; HDL, high-density lipoprotein; M, male; NGSP, National Glycohemoglobin Standardization Program.

*Smoking index is defined as packs per day × year. TAI and smoking index indicate the sum before and after first consultation.

†Outbreak of IFN monotherapy: recombinant IFN-α2a, n = 220, recombinant IFN-α2b, n = 183, natural IFN-α, n = 1,678, natural IFN-α, n = 691, total dose of IFN = 560 ± 164 megaunit. Outbreak of pegylated IFN monotherapy: pegylated IFN-α2a, n = 89, total dose of pegylated IFN = 7.52 ± 2.24 mg.

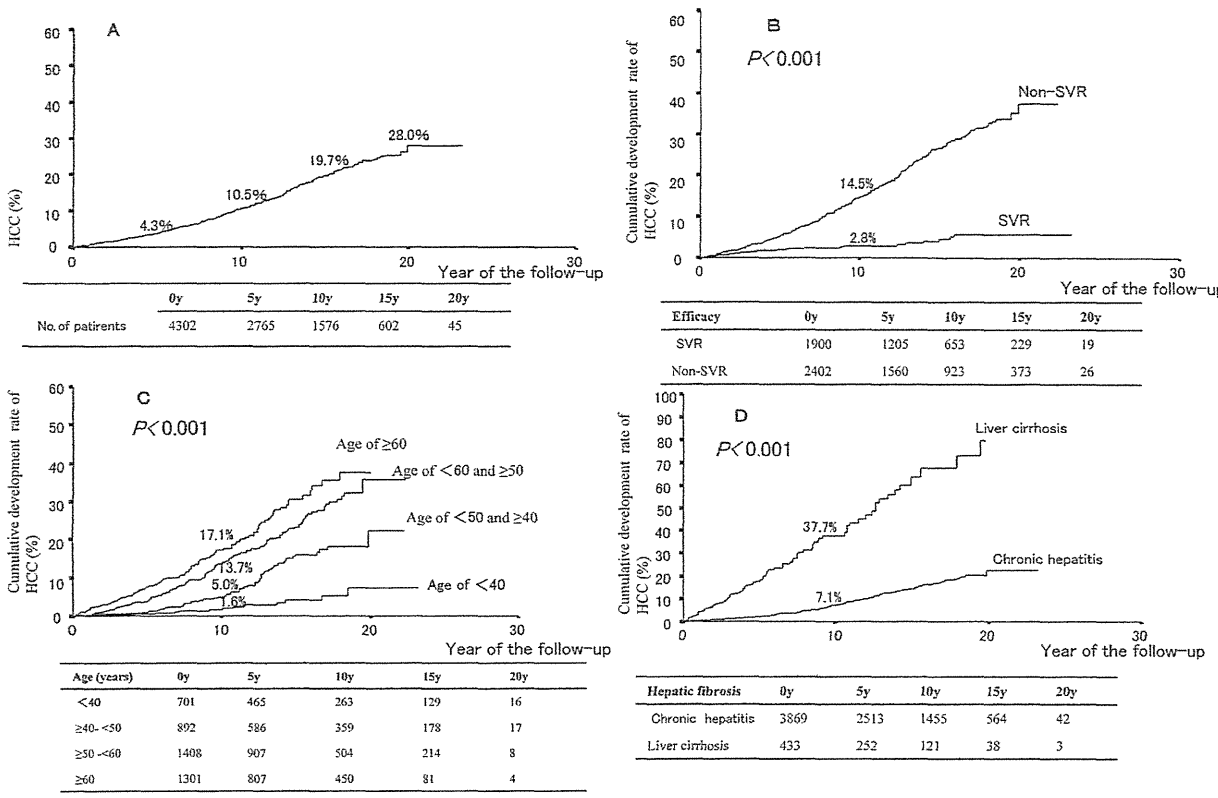
‡Outbreak of combination therapy: recombinant IFN-α2b + ribavirin, n = 335, total dose of IFN = 508 ± 184 megaunit, total dose of ribavirin = 160 ± 68 g; natural IFN-β + ribavirin, n = 101, total dose of IFN = 502 ± 176 megaunit, total dose of ribavirin = 156 ± 67 g; pegylated IFN-α2b+ribavirin, n = 1,005 cases, total dose of pegylated IFN = 4.14 ± 1.10 mg, total dose of ribavirin = 206 ± 58 g.

mL, and the undetectable samples were defined as negative. A sustained virological response (SVR) was defined as clearance of HCV-RNA using the COBAS TaqMan HCV test 6 months after the cessation of IFN therapy.

Evaluation of Liver Cirrhosis. Status of liver was mainly determined on the basis of peritoneoscopy and/or liver biopsy. Liver biopsy specimens were obtained using a modified Vim Silverman needle with an internal diameter of 2 mm (Tohoku University style; Kakinuma Factory, Tokyo, Japan), fixed in 10% formalin, and stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. The size of specimens for examination was more than six portal areas.[18]

Follow-up. The observation starting point was 6 months after the termination of IFN therapy. After that, patients were followed up at least twice a year in our hospital. Physical examination and biochemical tests were conducted at each examination together with a regular checkup. In addition, annual examinations during follow-up were undertaken. When a patient had complaints during follow-up, the physician in charge performed additional examinations based on symptoms. Four hundred eighteen patients were lost to follow-up. The final date of follow-up in 418 patients with loss of follow-up was regarded as the last consulting day. In addition, 881 patients were retreated with IFN. The final date of follow-up in 881 patients re-treated with IFN were regarded as the time of the initiation of IFN retreatment. Thus, 418 patients with loss of follow-up and 881 patients retreated with IFN were counted censored data in statistical analysis.[19] The mean follow-up period was 6.8 (SD 4.3) years in 418 patients with loss of follow-up and 7.5 (SD 4.8) years in 881 patients retreated with IFN. Censored patients were counted in the analysis.

Statistical Analysis. Clinical differences among three groups of patients with HCC with malignancies other than HCC without events were evaluated using the Kruskal-Wallis test. The cumulative development rates of malignancies were calculated using the Kaplan-Meier technique, and differences in the curves were



	0y	5y	10y	15y	20y
No. of patirents	4302	2765	1576	602	45

Efficacy	0y	5y	10y	15y	20y
SVR	1900	1205	653	229	19
Non-SVR	2402	1560	923	373	26

Age (years)	0y	5y	10y	15y	20y
<40	701	465	263	129	16
≥40-<50	892	586	359	178	17
≥50-<60	1408	907	504	214	8
≥60	1301	807	450	81	4

Hepatic fibrosis	0y	5y	10y	15y	20y
Chronic hepatitis	3869	2513	1455	564	42
Liver cirrhosis	433	252	121	38	3

Fig. 1. Cumulative development rate of HCC (A) in total HCV patients treated with IFN therapy and based on the difference of (B) efficacy, (C) age, and (D) hepatic fibrosis.

tested using the log-rank test.[20,21] Independent risk factors associated with malignancies were studied using the stepwise Cox regression analysis.[22] The following variables were analyzed for potential covariates for incidence of primary outcome: (1) age, sex, T2DM, and hypertension at the initiation time of follow-up; (2) HCV genotype, HCV load, and hepatic fibrosis before IFN therapy; (3) average value of body mass index, aspartate aminotransferase, alanine aminotransferase, triglyceride, total cholesterol, and platelet count during follow-up; (4) sum value of smoking and alcohol before, during, and after the IFN therapy; and (5) efficacy of IFN therapy, combination of ribavirin, type of IFN, and total dose of IFN. A $P < 0.05$ was considered statistically significant. Data analysis was performed using SPSS 11.5 for Windows (SPSS, Chicago, IL).

Results

Patient Characteristics. Table 1shows the baseline characteristics of the 4,302 enrolled patients at initiation of follow-up. The patients were divided into three groups: with HCC, with malignancies other than HCC, and without events. There were significant differences in several baseline characteristics among the three groups. The SVR rate was 34.4% (985/2,861) in IFN monotherapy and 63.5% (915/1,441) in combination therapy of IFN and ribavirin. Thus, the number of patients with SVR was 1,900. The mean follow-up was 8.1 (SD 5.0) years.

Development and Breakdown of Malignancies. As shown in Table 1, 606 of 4,302 patients developed malignancies: 393 developed HCC and 213 developed malignancies other than HCC. HCC accounted for 33.3% (44/132) of malignancies in patients with SVR and 73.6% (349/474) in patients without SVR. The breakdown of malignancies other than HCC was as follows: stomach cancer, n = 36; colon cancer, n = 35; lung cancer, n = 20; malignant lymphoma, n = 19; pancreatic cancer, n = 12; prostatic cancer, n = 16; breast cancer, n = 15; other cancers, n = 60.

Predictive Factors for the Development of HCC. The cumulative development rate of HCC was 4.3% at 5 years, 10.5% at 10 years, 19.7% at 15 years, and 28.0% at 20 years (Fig. 1A). The factors associated with the development of HCC are shown in Table 2. Multivariate Cox proportional hazards analysis

Table 2. Predictive Factors for Development of HCC in Enrolled Patients

	Univariate Analysis		Cox Regression Analysis	
Variable	HR (95% CI)	P	HR (95% CI)	P
Age, years (per 10)	1.84 (1.64-2.06)	<0.001	1.97 (1.71-2.28)	<0.001
Sex, male/female	1.47 (1.18-1.83)	<0.001	1.67 (1.24-2.23)	0.001
BMI, ≥22/<22	1.37 (1.12-1.66)	0.002		
T2DM, +/−	2.77 (2.13-3.60)	<0.001	1.73 (1.30-2.30)	<0.001
Hypertension, +/−	1.32 (1.02-1.71)	0.036		
Smoking index, ≥20/<20*	1.43 (1.14-1.79)	0.002		
TAI, kg, ≥200/<200*	2.13 (1.74-2.61)	<0.001	1.45 (1.11-1.88)	0.007
AST, IU/L, ≥34/<34	3.00 (2.40-3.89)	<0.001		
ALT, IU/L, ≥36/<36	2.74 (2.16-3.42)	<0.001		
GGT, IU/L, ≥109/<109	1.79 (1.19-2.46)	0.039		
Albumin, g/dL, <3.9/≥3.9	1.92 (1.37-2.55)	0.015		
Triglyceride, mg/dL, ≥100/<100	1.14 (0.94-1.37)	0.179		
Cholesterol, mg/dL, <150/≥150\	1.38 (1.10-1.72)	0.004		
Platelet count, $\times 10^4/mm^3$, <15/≥15)	3.27 (2.56-4.17)	<0.001		
Histological diagnosis, LC/non-LC	7.09 (5.59-9.01)	<0.001	5.01 (3.92-6.40)	<0.001
Combination of ribavirin, +/−	0.66 (0.45-0.97)	0.033		
Type of IFN, α/β	1.10 (0.85-1.41)	0.474		
Total dose of IFN, MU, ≥500/<500	1.12 (0.91-1.38)	0.291		
HCV genotype, ½	1.67 (1.30-2.14)	<0.001		
HCV-RNA, log IU/mL, ≥5/<5	1.02 (0.98-1.05)	0.315		
Efficacy, non-SVR/SVR	4.78 (3.47-6.59)	<0.001	4.93 (3.53-6.89)	<0.001

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; GGT, gamma-glutamyl transferase; HDL, high-density lipoprotein.

*Smoking index is defined as packs per day × year. TAI and smoking index indicate the sum before and after first consultation.

showed that HCC occurred when patients had liver cirrhosis (hazard ratio [HR], 5.01; 95% confidence interval [CI], 3.92-6.40; $P < 0.001$), non-SVR (HR, 4.93; 95% CI, 3.53-6.89; $P < 0.001$), age increments of 10 years (HR, 1.97; 95% CI, 1.71-2.28; $P < 0.001$), T2DM (HR, 1.73; 95% CI, 1.30-2.30; $P < 0.001$), male sex (HR, 1.67; 95% CI, 1.24-2.23; $P = 0.001$), and TAI of ≥ 200 kg (HR, 1.45; 95% CI, 1.11-1.88; $P = 0.007$). Fig. 1B-D and Fig. 2A-C show the cumulative development rates of HCC based on difference of IFN efficacy, age, hepatic fibrosis, TAI, sex, and T2DM. The 10-year cumulative rates of HCC after IFN therapy was determined to be 7.1% in 3,869 patients with chronic hepatitis and 37.7% in 433 patients with cirrhosis by using the Kaplan-Meier Method (Fig. 1D). Fig. 2D shows the development rates of HCC in T2DM patients according to difference of mean hemoglobin A1c (HbA1c) level during follow-up. HCC decreased when T2DM patients had a mean HbA1c level of <7.0% during follow-up (HR, 0.56; 95% CI, 0.33-0.89; $P = 0.015$). The development of HCC was reduced by 44% in T2DM patients with a mean HbA1c level of <7.0% compared with those with a mean HbA1c level of ≥7.0%.

Table 3 shows the development rate of HCC and risk factors in four groups classified by the difference of hepatic fibrosis and efficacy of IFN therapy. The development rate of HCC per 1,000 person years was 1.55 in patients with chronic hepatitis (CH) at baseline and SVR (CH+SVR), 18.23 in patients with liver cirrhosis (LC) at baseline and SVR (LC+SVR), 13.53 in patients with chronic hepatitis at baseline and non-SVR (CH+non-SVR), and 50.43 in patients with LC at baseline and non-SVR (LC+non-SVR). The risk of HCC development in the CH+SVR group was advanced age, male sex, TAI of ≥200 kg, and T2DM. T2DM enhanced the development of HCC with statistical significance in three groups of CH+SVR, CH+non-SVR, and LC+non-SVR.

Predictive Factors for Development of Malignancies Other than HCC. The cumulative development rate of malignancies other than HCC was 2.4% at 5 years, 5.1% at 10 years, 9.8% at 15 years, and 18.0% at 20 years (Fig. 3A). The factors associated with the development of malignancies other than HCC are shown in Table 4. Malignancies other than HCC occurred when patients had age increments of 10 years (HR, 2.19; 95% CI, 1.84-2.62; $P < 0.001$), smoking index of ≥20 (HR, 1.89; 95% CI, 1.41-2.53; $P < 0.001$), and T2DM (HR, 1.70; 95% CI, 1.14-2.53; $P = 0.008$). Fig. 3B-D shows the cumulative development rates of malignancies other than HCC based on difference of age, smoking index, and T2DM. Fig. 3E shows the risk of malignancies other than HCC in T2DM patients according to mean HbA1c level during follow-up. The HR of HCC development in

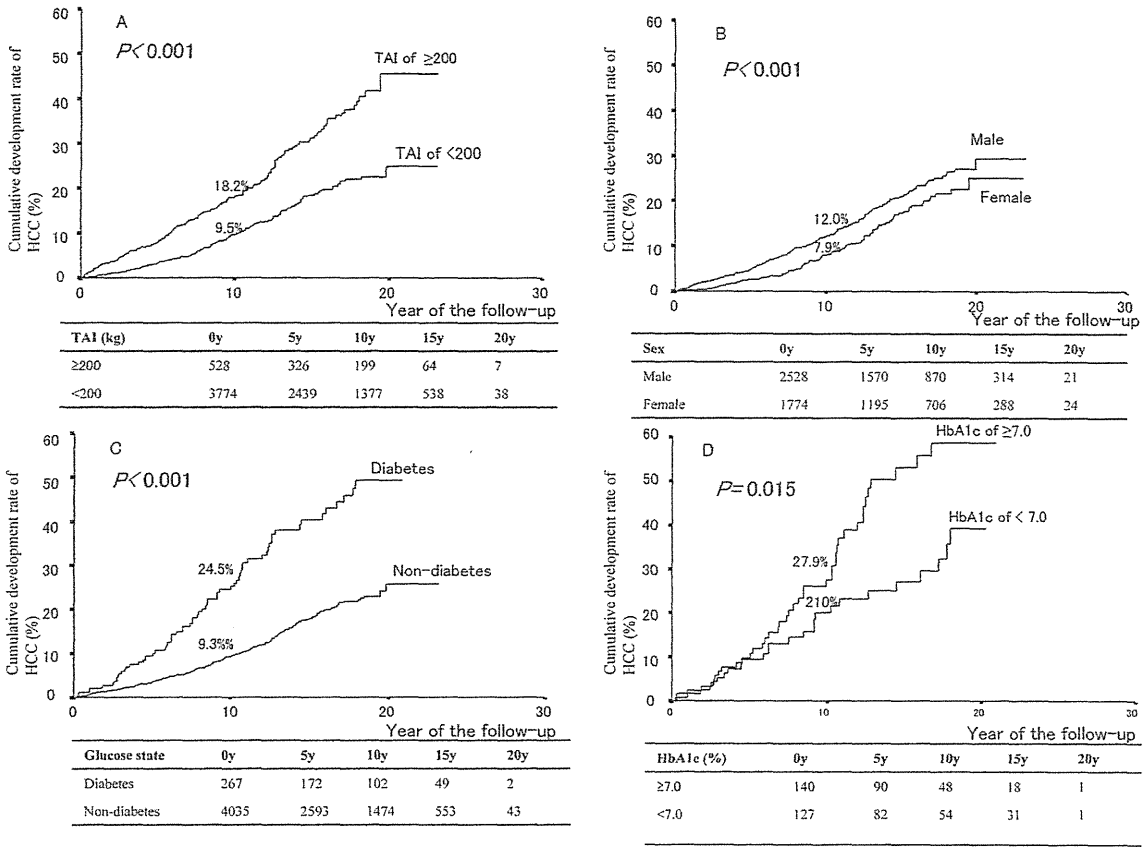


Fig. 2. Cumulative development rate of HCC based on the difference of (A) TAI, (B) sex, (C) diabetic state, and (D) mean HbA1c level during follow-up in T2DM patients.

patients with mean HbA1c level of <7.0% versus those with mean HbA1c level of ≥7.0% was 0.62 (95% CI, 0.31-1.23; $P = 0.170$). There was no significant difference in development of malignancies other than HCC based on the difference of mean HbA1c level during follow-up. Table 5 shows the impact based

Table 3. Development Rate of HCC Based on Hepatic Fibrosis and Efficacy of IFN Therapy

Variable	CH + SVR	LC + SVR	CH + Non-SVR	LC + Non-SVR
No. of patients	1,751	149	2,118	284
Age, years	51.7 ± 12.1	56.9 ± 9.8	51.5 ± 11.7	57.2 ± 9.9
Sex, male/female	1,082/669	91/58	1,190/928	165/119
HbA1c (%, NSPG)	5.5 ± 0.7	5.8 ± 0.8	5.7 ± 0.7	6.1 ± 0.8
TAI, kg	86 ± 91	104 ± 99	97 ± 90	129 ± 102
Patients with T2DM	74	13	133	47
Patients with HCC	22	22	233	116
1,000 person years of HCC	1.55	18.23	13.53	50.43
Age, years (per 10)*	2.60 (1.48-4.58)	1.83 (0.95-3.55)	2.07 (1.75-2.46)	1.09 (0.87-1.37)
P value	0.001	0.070	<0.001	0.477
Sex, male/female*	3.42 (1.01-11.63)	3.41 (1.00-11.63)	1.34 (0.99-1.81)	1.93 (1.25-3.00)
P value	0.049	0.050	0.058	0.003
TAI, kg, ≥200/<200*	2.68 (1.14-6.34)	3.84 (1.83-9.85)	2.21 (1.65-2.95)	1.54 (1.03-2.31)
P value	0.024	0.004	<0.001	0.038
T2DM, +/−*	4.76 (1.60-14.10)	2.48 (0.57-10.86)	2.53 (1.76-3.65)	1.87 (1.16-3.01)
P value	0.005	0.228	<0.001	0.010

Abbreviations: CH + Non-SVR, patients with CH at baseline and non-SVR 6 months after IFN therapy; CH + SVR, patients with CH at baseline and SVR 6 months after IFN therapy; LC + Non-SVR, patients with LC at baseline and non-SVR 6 months after IFN therapy; LC + SVR, patients with LC at baseline and SVR 6 months after IFN therapy.

*Hazard ratio (95% confidence interval) and *P* value by Cox proportional hazards analysis.

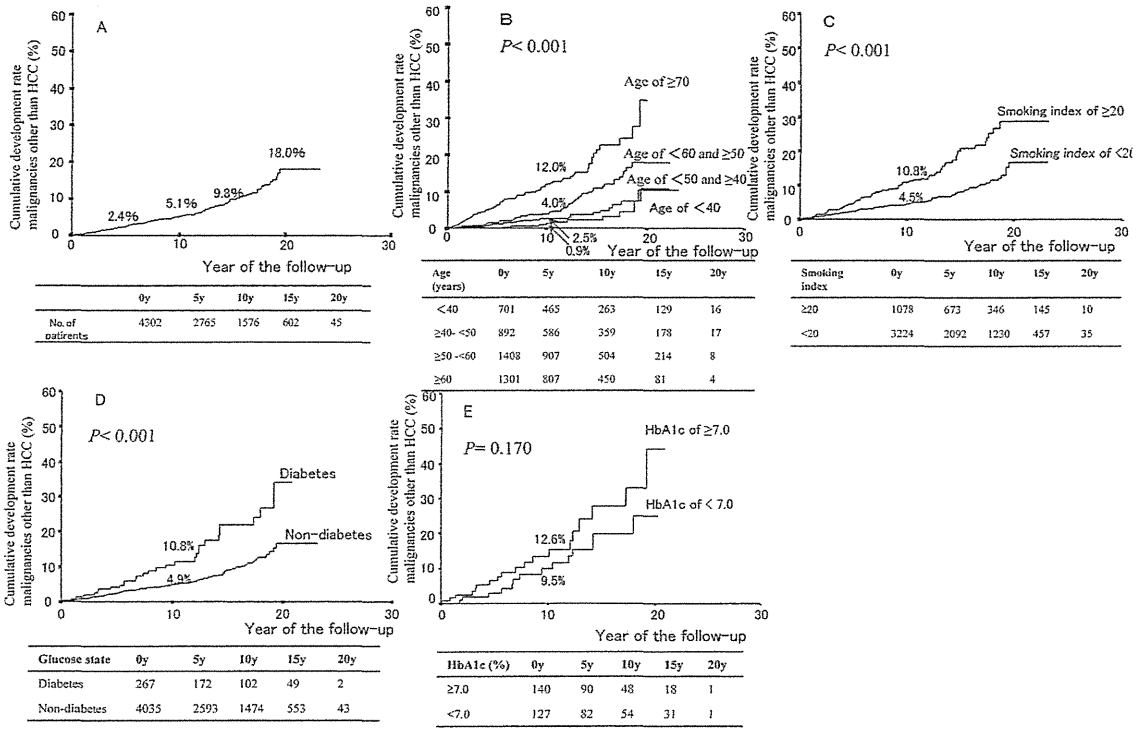


Fig. 3. Cumulative development rate of malignancies other than HCC (A) in total HCV patients treated with IFN therapy and based on the difference of (B) age, (C) smoking index, (D) diabetic state, and (E) mean HbA1c level during follow-up in T2DM patients.

on three factors of age, smoking index, and T2DM for the development of each malignancy other than HCC by using Cox regression analysis. Aging enhanced carcinogenesis of stomach, colon, lung, prostate, breast, and pancreas with statistical significance. Smoking enhanced lung cancer and colorectal cancer

Table 4. Predictive Factors for Development of Malignancies Other than HCC

	Univariate Analysis		Cox-Regression Analysis	
Variables	HR (95% CI)	P	HR (95% CI)	P
Age, years (per 10)	2.23 (1.88-2.65)	< 0.001	2.19 (1.84-2.62)	<0.001
Sex, male/female	1.06 (0.79-1.40)	0.759		
BMI, ≥22/<22	0.97 (0.75-1.24)	0.767		
T2DM, +/−	2.56 (1.76-3.72)	<0.001	1.70 (1.14-2.53)	0.008
Hypertension, +/−	2.33 (1.70-3.18)	<0.001		
Smoking index, ≥20/<20*	2.74 (2.06-3.65)	<0.001	1.89 (1.41-2.53)	<0.001
TAI, kg, ≥200/<200*	1.77 (1.33-2.37)	<0.001		
AST, IU/L, ≥34/<34	0.89 (0.65-1.20)	0.412		
ALT, IU/L, ≥36/<36	0.98 (0.72-1.34)	0.891		
GGT, IU/L, ≥109/<109	1.26 (0.79-2.01)	0.350		
Albumin, g/dL, <3.9/≥3.9	1.41 (0.90-2.04)	0.145		
Triglyceride, mg/dL, ≥100/<100	1.28 (1.03-1.60)	0.030		
Total cholesterol, mg/dL, <150/≥150	1.10 (0.82-1.46)	0.548		
Platelet count, × $10^4/mm^3$, <15/≥15	1.39 (1.02-1.91)	0.038		
Histological diagnosis, LC/non-LC	1.77 (1.13-2.75)	0.012		
Combination of ribavirin, +/−	0.66 (0.44-0.97)	0.034		
Type of IFN, α/β	1.05 (0.75-1.47)	0.789		
Total dose of IFN, MU, ≥500/<500	1.31 (0.96-1.77)	0.084		
HCV genotype, ½	1.30 (0.80-2.93)	0.432		
HCV RNA, log IU/mL, ≥5/<5	0.89 (0.50-1.23)	0.612		
Efficacy, non-SVR/SVR	0.85 (0.64-1.12)	0.232		

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; GGT, gamma-glutamyl transferase.
*Smoking index is defined as packs per day × year. TAI and smoking index indicate the sum before and after first consultation.

Table 5. Impact Based on Age, Smoking Index, and Diabetes for Development of Malignancies Other than HCC

	Age, Years (per 10)		Smoking Index, ≥20/<20		Diabetes, +/−	
Malignancy	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
Gastric cancer (n = 36)	2.48 (1.62-3.78)	<0.001	1.69 (0.83-3.43)	0.146	2.29 (0.95-5.52)	0.065
Colorectal cancer (n = 35)	1.91 (1.28-2.86)	0.002	2.27 (1.13-4.58)	0.022	1.78 (0.68-4.66)	0.240
Lung cancer (n = 20)	2.33 (1.35-4.01)	0.002	2.90 (1.25-6.74)	0.013	1.53 (0.45-5.24)	0.496
Prostatic cancer (n = 16)	2.84 (1.32-6.13)	0.008	1.89 (0.88-3.15)	0.266	0.71 (0.09-5.47)	0.735
Breast cancer (n = 15)	2.86 (1.30-6.29)	0.009	1.29 (0.17-10.19)	0.808	1.20 (0.16-9.39)	0.859
Malignant lymphoma (n = 19)	2.21 (1.26-3.88)	0.006	1.25 (0.44-3.56)	0.671	1.39 (0.32-6.12)	0.663
Pancreatic cancer (n = 12)	3.32 (1.44-7.65)	0.005	1.41 (0.45-4.82)	0.578	3.75 (1.02-13.88)	0.046

with statistical significance. In addition, T2DM enhanced the pancreatic cancer with statistical significance and tended to enhance the gastric cancer.

Discussion

This study describes the development incidence of HCC or malignancies other than HCC after the termination of IFN therapy in HCV patients. Patients at Toranomon Hospital comprised mainly government employees, office workers, and business persons. Most patients were regularly recommended to undergo annual multiphasic health screening examinations. In the present study, patients who had undergone annual multiphasic health screening examinations were enrolled. The strengths of the present study are a prolonged follow-up in the large numbers of patients included.

The present study shows several findings with regard to the development incidence and predictive factors for total malignancies after IFN therapy for HCV patients. First, the 10-year cumulative rates of HCC after IFN therapy was determined to be 7.1% in 3,869 patients with chronic hepatitis and 37.7% in 433 patients with cirrhosis using the Kaplan-Meier method. Our previous studies showed via retrospective analysis that the 10-year cumulative rates of HCC were 12.4% for 456 patients with chronic hepatitis and 53.2% for 349 patients with cirrhosis.[7,23] Although patient selection bias for IFN treatment versus no treatment had been noted in the previous studies, the results suggest the possibility that IFN therapy reduces the development of HCC in HCV patients. Several historical data in Japan suggest that IFN therapy reduces the development of HCC in HCV patients.[24-26]

Second, HCC occurred with statistical significance when the following characteristics were present: non-SVR, advanced age, cirrhosis, TAI of ≥200 kg, male sex, and T2DM. T2DM caused a 1.73-fold enhancement in HCC development. Several authors have reported an increased risk of HCC among patients with the following characteristics: non-SVR, cirrhosis, male sex, advanced age, and T2DM.[24-28] Our results show that physicians in charge of aged male patients with non-SVR, advanced fibrosis, TAI of ≥200 kg, and T2DM should pay attention to the development of HCC after IFN therapy. In addition, maintaining a mean HbA1c level of <7.0% during follow-up reduced the development of HCC. This result indicates that stringent control of T2DM is important for protecting the development of HCC.

Third, the development rate of HCC per 1,000 person years was about 1.55 in 1,751 patients with chronic hepatitis at baseline and SVR. In these patients, the risk factors associated with HCC were advanced age, male sex, TAI, and T2DM. We compared the HCC development rate in patients with chronic hepatitis at baseline and SVR to the general population. A total of 5,253 individuals without HCV antibody and hepatitis B surface antigen, who underwent annual multiphasic health screening examinations in our hospital were evaluated as controls. Individuals with either of the following criteria were excluded: (1) illness that could seriously reduce their life expectancy or (2) history of carcinogenesis. They were selected by matching 3:1 with patients who had chronic hepatitis at baseline and SVR for age, sex, T2DM, and follow-up periods. In control individuals, the mean age was 51.7 years; the prevalence (number) of male patients was 61.8% (3,246); the prevalence (number) of T2DM patients was 4.2% (222); the mean follow-up period was 8.0 years. The number of development of HCC in control individuals was only five. This result suggests that the development rate of HCC in patients with chronic hepatitis at baseline and SVR is higher than that in the general population.

Fourth, HCC accounted for 33.3% in SVR patients and 73.6% in non-SVR patients. According to Matsuda et al.,[29] the outbreak of malignancies in the Japanese male population was observed in the following order in 2005: gastric cancer 20.4% > colon

cancer 16.0% > lung cancer 15.4% > prostate cancer 10.9% > HCC 7.4%. On the other hand, the outbreak of malignancies in the Japanese female population was observed in the following order in 2005: breast cancer 18.0% > colon cancer 16.2% > gastric cancer 13.6% > lung cancer 9.3% > uterine cancer 6.8%. Our results show that HCC is the most common cause of malignancy, not only in the non-SVR group but also in the SVR group.

Finally, malignancies other than HCC occurred with statistical significance when patients were of advanced age, were smokers, and had T2DM. Our result indicates that smoking enhances lung cancer and colorectal cancer. Many authors have reported that smoking is a direct cause of cancers of the oral cavity, esophagus, stomach, pancreas, larynx, lung, bladder, kidney, and colon.[30,31] In addition, the present study indicates that T2DM enhances pancreatic cancer with statistical significance and tends to enhance gastric cancer. T2DM showed up to about 1.7-fold increase in development of malignancies other than HCC. A recent meta-analysis of cohort studies have revealed that diabetic patients increase risk of pancreatic cancer, HCC, bladder cancer, non-Hodgkin's lymphoma, colorectal cancer, and breast cancer.[32-39]

Although the role of T2DM in carcinogenesis remains speculative, the following possible mechanisms have been reported: (1) hyperglycemia increases malignancy risk via increasing oxidative stress and/or activating the rennin-angiotensin system[40]; (2) insulin resistance increases malignancy risk via down-regulation of serine/threonine kinase II to adenosine monophosphate–activated protein kinase pathway[41]; (3) reduced insulin secretion increases malignancy risk via down-regulation of sterol regulatory element-binding protein-1c with consequent up-regulation of insulin-like growth factor.[42]

T2DM is increasing dramatically worldwide over the past decades.[8] It is estimated that about 7 million people are affected by diabetes mellitus in Japan. Approximately 8%-10% of adults in Japan have T2DM. The risk factors associated with T2DM include family history, age, sex, obesity, smoking, physical activity, and HCV.[43-46] In the near future, T2DM will be increasing in HCV-positive patients.

This study is limited in that it was a retrospective cohort trial. Another limitation is that patients were treated with different types of antivirus therapy for different durations. In addition, T2DM patients were treated with different types of drugs during follow-up. Finally, our cohort contains Japanese subjects only. On the other hand, the strengths of the present study are a long-term follow-up in the large numbers of patients included.

In conclusion, T2DM causes an approximately 1.7-fold enhancement in the development of HCC and malignancies other than HCC after IFN therapy. Additionally, in T2DM patients, maintaining a mean HbA1c level of <7.0% during follow-up reduced the development of HCC.

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Research Article

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Characterization of virologic escape in hepatitis C virus genotype 1b patients treated with the direct-acting antivirals daclatasvir and asunaprevir

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See Editorial, pages 643–645

Background & Aims: Daclatasvir and asunaprevir are NS5A and NS3 protease-targeted antivirals currently under development for treatment of chronic hepatitis C virus infection. Clinical data on baseline and on-treatment correlates of drug resistance and response to these agents are currently limited.

Methods: Hepatitis C virus genotype 1b Japanese patients (prior null responders to PegIFN-α/RBV [n = 21] or PegIFN-α/RBV ineligible or intolerant [n = 22]) were administered daclatasvir/asunaprevir for 24 weeks during a phase 2a open-label study. Genotypic and phenotypic analyses of NS3 and NS5A substitutions were performed at baseline, after virologic failure, and post-treatment through follow-up week 36.

Results: There were three viral breakthroughs and four relapsers. Baseline NS3 polymorphisms (T54S, Q80L, V170M) at amino acid positions previously associated with low-level resistance (<9-fold) to select NS3 protease inhibitors were detected in four null responders and three ineligibles, but were not associated with virologic failure. Baseline NS5A polymorphisms (L28M, L31M, Y93H) associated with daclatasvir resistance (<25-fold) were detected in five null responders and six ineligibles. All three viral breakthroughs and 2/4 relapsers carried a baseline NS5A-Y93H polymorphism. NS3 and NS5A resistance-associated variants were detected together (NS3-D168A/V, NS5A-L31M/V-Y93H) after virologic failure. Generally, daclatasvir-resistant substitutions persisted through 48 weeks post-treatment, whereas asunaprevir-resistant substitutions were no longer detectable. Overall, 5/10 patients with baseline NS5A-Y93H experienced virologic failure, while 5/10 achieved a sustained virologic response.

Conclusions: The potential association of a pre-existing NS5A-Y93H polymorphism with virologic failure on daclatasvir/asunaprevir combination treatment will be examined in larger studies. The persistence of treatment-emergent daclatasvir- and asunaprevir-resistant substitutions will require assessment in longer-term follow-up studies.

Keywords: Asunaprevir; Daclatasvir; Drug resistance; Direct-acting antivirals; Hepatitis C; Peginterferon-sparing.

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Abbreviations: DAA, direct-acting antiviral; HCV, hepatitis C virus; SVR, sustained virologic response; GT, genotype; PegIFN-α/RBV, peginterferon alfa and ribavirin; DCV, daclatasvir; ASV, asunaprevir; LLOQ, lower limit of quantitation; PCR, polymerase chain reaction; FU, follow-up; RAV, resistance-associated variant; BL, baseline; VBT, viral breakthrough; SD, standard deviation.

Introduction

The introduction of direct-acting antivirals (DAAs) targeting hepatitis C virus (HCV) NS3 protease activity has substantially increased sustained virologic response (SVR) in chronic HCV genotype 1 (GT1) infection. In combination with peginterferon-alfa and ribavirin (PegIFN-α/RBV), treatment with the recently approved protease inhibitors boceprevir or telaprevir results in SVR rates of around 70–75% in treatment-naive patients [1,2]. Despite these improvements, SVR rates vary by genotype and remain suboptimal in some patients, such as null responders to PegIFN-α/RBV [3], and patients for whom PegIFN-α/RBV is poorly tolerated or medically contraindicated. Furthermore, PegIFN-α/RBV is associated with frequent side effects [3], and the addition of these DAAs results in elevated rates of anemia and additional events such as dysgeusia (boceprevir), or rash, pruritus, and nausea (telaprevir) [4,5].

Daclatasvir (DCV) and asunaprevir (ASV) are currently undergoing clinical development for HCV infection. DCV (BMS-790052) is a first-in-class, highly selective NS5A replication complex inhibitor with picomolar potency and broad HCV genotypic coverage [6] that has demonstrated antiviral efficacy and good tolerability in combination with PegIFN-α/RBV [7]. ASV (BMS-650032) is a selective inhibitor of NS3 protease with antiviral activity *in vitro* against GT1 and GT4 [8]; it has also been shown to be

efficacious and generally well tolerated in combination with PegIFN-α/RBV [9]. Clinical interest is increasingly focusing on exploring DAA-only regimens without PegIFN-α/RBV, whose potential benefits might include better tolerability and compliance, and a reduced duration of therapy. One recent PegIFN-α/RBV-sparing study of DCV plus ASV (AI447017) has examined the efficacy and safety of this combination for 24 weeks in a small cohort of ten GT1b null responders, in whom an SVR rate of 90% was observed [10]. The study was then expanded to include an additional cohort of null responders and a group of patients ineligible to receive, or intolerant of, PegIFN-α/RBV [11].

As with other antiviral agents, the efficacy of DCV and ASV can be compromised by the development of drug resistance. *In vitro* data suggest that DCV and ASV should provide additive or synergistic activity that enhances the genetic barrier to resistance [8]. Here we characterize virologic escape observed on DCV plus ASV treatment in the expanded AI447017 study [11]; its associations with baseline characteristics, including *IL28B* genotype and HCV polymorphisms; and an assessment of on- and off-treatment genotypic changes in NS5A and NS3 protease and their phenotypic consequences.

Patients and methods

Study design and patients

This was an open-label, phase 2a study (AI447017; clinicaltrials.gov identifier NCT01051414) evaluating the antiviral activity and safety of DCV plus ASV in 43 patients with HCV GT1 infection. Patients comprised (a) 21 PegIFN-α/RBV null responders (<2 $\log_{10}$ decline in plasma HCV RNA after 12 weeks) and (b) 22 patients who discontinued previous PegIFN-α/RBV within 12 weeks for intolerance or were considered medically poor candidates for PegIFN-α/RBV for reasons such as advanced age, complications of depression, anemia, myelosuppression, diabetes, or cardiovascular or renal dysfunction. Patients enrolled in four cohorts; two each of null responders and ineligible/intolerant patients. The initial sentinel cohort of null responders has been described previously [10]. All enrolled patients were infected with GT1b.

Patients received DCV 60 mg once daily with ASV 200 mg twice daily for 24 weeks, with a further 48 weeks of post-treatment follow-up. ASV dosing in the expanded study was reduced from the 600 mg twice-daily administration used in the sentinel cohort, following reports of hepatic enzyme elevations at this dose, in another clinical study [12].

The full study design, including inclusion/exclusion criteria, and safety/efficacy endpoints, is described elsewhere [11]. Briefly, eligible patients were men and women aged 20–75 years with HCV GT1 infection ⩾6 months and HCV RNA ⩾10^5 IU/ml. Patients were excluded if they had evidence of liver cirrhosis within 24 months of screening; a history of hepatocellular carcinoma, other chronic liver disease, variceal bleeding, hepatic encephalopathy, or ascites requiring diuretics or paracentesis; co-infection with hepatitis B virus or HIV; or other clinically significant medical conditions.

Laboratory assessments

Plasma samples for resistance testing were collected at baseline and study weeks 1, 2, 4, 6, 8, 10, 12, 16, 20, and 24 and post-treatment weeks 4, 8, 12, 24, 36, and 48. HCV RNA was determined at a central laboratory using the Roche COBAS® TaqMan® HCV Auto assay (Roche Diagnostics KK, Tokyo, Japan) with a lower limit of quantitation (LLOQ) of 15 IU/ml. HCV genotype and subtype and *IL28B* genotype (rs12979860 single-nucleotide polymorphism) were determined by polymerase chain reaction (PCR) amplification and sequencing.

Genotypic and phenotypic analysis of clinical samples

Testing was performed on all baseline samples and on samples indicative of slow virologic response at week 1 or virologic failure with HCV RNA levels ⩾1000 IU/ml. Virologic failure, for the purpose of the study, was defined as an HCV RNA level (a) ⩾LLOQ at week 4 (futility rule), (b) >1 $\log_{10}$ IU/ml above nadir or ⩾LLOQ after confirmed undetectable (virologic breakthrough), or (c) ⩾LLOQ at any follow-up visit after being undetectable at the end of treatment (relapse).

Population sequencing of PCR amplicons was performed using methods described elsewhere [13–15]. For clonal analysis, amplicons were cloned into the TOPO vector and transformed into TOP10 *Escherichia coli* using a commercially available kit (TOPO® TA-cloning® kit, Invitrogen, Carlsbad, CA) according to manufacturer's instructions, with ⩾20 individual colonies expanded and sequenced for each analysis.

Phenotypic analyses of resistance-associated substitutions were performed by employing *in vitro* HCV replicon systems according to previously published methodologies [15–17].

Results

Viral response to DCV and ASV

Overall, plasma HCV RNA was undetectable in 77% (33/43) of patients at 24 weeks post-treatment. SVR was higher among the null responders than in the PegIFN-α/RBV ineligible population; all viral breakthroughs (n = 3) and relapses (n = 4) occurred in the ineligible/intolerant subpopulation. Three patients discontinued the study without subsequent SVR or virologic failure (Tables 1 and 2) [11].

Null responders

Virologic response.

Rapid and similar decreases in plasma HCV RNA levels were observed among patients who initiated treatment with ASV 600 mg (Fig. 1A) or ASV 200 mg (Fig. 1B). Mean reduction in HCV RNA at week 1 was comparable for both groups (−4.4 *vs.* −4.3 $\log_{10}$ IU/ml, respectively). Of the patients still receiving treatment (P-6 discontinued at day 16 due to an AE), all but one patient (P-13) had HCV RNA <15 IU/ml at week 4 and 52% had undetectable HCV RNA at this time.

Baseline analysis. Baseline *IL28B* genotype and naturally occurring polymorphisms associated with ASV or DCV resistance (resistance-associated variants [RAVs]) are shown in Table 1. As anticipated for this prior null responder population, the majority (18/21) were non-CC *IL28B*. The NS5A polymorphism Y93H (24-fold DCV resistance [13]) was observed in three patients. Other polymorphisms conferring minimal (two- to three-fold) DCV resistance were detected in two patients (NS5A-L28M-R30Q and NS5A-L31M). Polymorphisms associated with minimal to low-level resistance to select NS3 protease inhibitors (one patient, NS3-T54S-Q80L; one patient, NS3-Q80L-V170I/M; two patients, NS3-Q80L) [4,5,18] were also observed.

Baseline polymorphisms and *IL28B* genotype did not appear to influence either the week 1 response or SVR rate (Fig. 2A). Five patients had RNA levels ⩾1000 IU/ml after 1 week, of whom one (P-21) had significantly slower initial HCV RNA declines when compared with mean reductions (standard deviation [SD]) in HCV RNA for null responders on the study (−3.4 *vs.* −4.35 ± 0.49 $\log_{10}$ IU/ml). This patient had a CC *IL28B* genotype and an NS5A polymorphism (Q54L; no fold-change in DCV resistance). The other four patients had polymorphisms that have been associated with DCV and NS3 protease inhibitor low-level resistance [13,19]—specifically NS5A-Q54H/Q-Q62Q/E-Y93H/Y with NS3-T54S-Q80L (P-1, no fold-change to DCV/ASV), NS3-Q80L-V170I/M (P-2, no fold-change to ASV), NS5A-R30Q

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Table 1. Baseline viral and host characteristics among genotype-1b null responders and their virologic outcome.

Patient	*IL28B* GT	HCV RNA, log_{10} IU/ml	NS5A polymorphism(s)[a]	NS3 polymorphism(s)[a]	Virologic outcome
P-1	CT	7.2	Q54H/Q-Q62Q/E-Y93H/Y	T54S-Q80L	SVR
P-2	CT	7.0		Q80L-V170I/M	SVR
P-3	CT	7.4	Q54H		SVR
P-4	CT	6.7	R30Q		SVR
P-5	CT	7.0	L31L/M-P58P/S		SVR
P-6	CC	5.3	P58P/T-Q62E		D/C at Wk2 due to SAE[b]
P-7	CC	7.2		S122S/G	SVR
P-8	CT	7.0	Q54H	Q80L	SVR
P-9	CT	7.1	Q54H-Y93H/Y	S122N	SVR
P-10	CT	6.4	L28M-R30Q		SVR
P-11	CT	6.8			D/C at Wk12 due to AE; SVR
P-12	CT	6.4	Q54H-P58S-Q62E		SVR
P-13	CT	7.4	Q54H		D/C at Wk6; PDR not achieved[c]
P-14	CT	6.5			SVR
P-15	CT	6.3	R30Q/R-Q62Q/R		SVR
P-16	CT	6.6	Q54H		SVR
P-17	CT	6.6	Q54H-Q62E		SVR
P-18	CT	6.9	Q54Y	Q80L	SVR
P-19	CT	6.6	Q54H-Y93H	N77A	SVR
P-20	CT	7.0	R30Q	S122G	SVR
P-21	CC	6.6	Q54L		SVR

[a]All NS3 and NS5A amino acids were examined with focus on polymorphisms at positions known to be associated with resistance to NS3 protease inhibitors (36, 43, 54, 55, 77, 78, 79, 80, 122, 123, 138, 155, 156, 158, 168, 170, 175) and NS5A inhibitors (21, 23, 24, 28, 30, 31, 32, 54, 58, 62, 92, 93). When a mixture of substitutions is indicated, the most predominant is identified first.
[b]HCV RNA undetectable at post-treatment week 24.
[c]PegIFN-α/RBV added; HCV RNA undetectable at post-treatment week 24 following 52 weeks of therapy.
AE, adverse event; D/C, discontinued; GT, genotype; HCV, hepatitis C virus; PDR, protocol-defined response; SAE, serious adverse event; SVR, sustained virologic response; Wk, week.

with NS3-S122G (P-20, no fold-change to either DCV/ASV), or NS5A-Q54H (P-13, no fold-change to DCV). P-13 was the only patient with HCV RNA <15 IU/ml (target detectable) at week 6 and was, therefore, considered a treatment failure. Treatment-emergent resistance at week 1 in the five patients could not be determined because of PCR failure. A comparison of initial virologic response *vs.* dose and polymorphisms associated with resistance revealed no differences. Among null responders who received ASV 600 mg, mean HCV RNA declines at week 1 for those with *vs.* without RAVs were −4.6 *vs.* −4.3 log_{10} IU/ml, which were similar to the week 1 declines among those who received ASV 200 mg (−4.5 log_{10} IU/ml with RAVs [one patient] *vs.* −4.3 log_{10}).

Baseline HCV RNA levels did not have an impact on response to treatment; patients with high baseline viral load still experienced rapid and robust responses to therapy (Fig. 1; Table 1).

Ineligible/intolerant patients

Virologic response.

Virologic response at week 4 was greater in PegIFN-α/RBV ineligible patients than in null responders. Undetectable HCV RNA at week 4 was observed in 86% of the ineligible group *vs.* 52% of null responders. However, by week 12, undetectable HCV RNA was similar in both groups. Early HCV RNA declines appeared unaffected by *IL28B* genotype, the presence of baseline polymorphisms associated with resistance, or virologic outcome (Fig. 3). Adherence to therapy, assessed through pill counts, was found to be high in six of the seven patients experiencing virologic failure. However, DCV/ASV exposures were high in the one non-compliant patient (P-31) who subsequently experienced relapse.

Baseline analysis.

Baseline *IL28B* genotype, polymorphisms associated with resistance, and virologic outcome are shown in Table 2 and Fig. 2B. Three patients presented with DCV resistance at baseline: one (P-25) with an NS5A-L31M-Y93H combination (7105-fold DCV resistance [13]) and two with an NS5A-Q54Y-Y93H (58-fold resistance). All three subsequently experienced viral breakthrough at week 10 or 16.

Other patients had baseline polymorphisms conferring minimal or low-level resistance to DCV and/or protease inhibitors; NS5A-Y93H (n = 4), NS5A-L28M-R30L (n = 1), NS3-T54S (n = 1), and NS3-Q80L (n = 5). Variable responses were observed among these patients (Fig. 2B); the majority responded, but two patients with baseline NS5A-Y93H experienced post-treatment relapse. One patient (P-24) with baseline NS5A-L28M-R30L-Q54H-A92T and NS3-Q80L-S122G had a slower response to treatment at week 1 when compared with mean HCV RNA reductions (SD) for ineligible/intolerant patients on the study (−3.4 *vs.* −4.74 [0.58] log_{10} IU/ml), but subsequently achieved SVR with only 16 weeks of treatment. Neither NS3-Q80L-S122G nor NS5A-L28M-R30L-Q54H-A92T conferred resistance to ASV or DCV, respectively.

Baseline viral load did not appear to affect response; mean HCV RNA levels (SD) were 6.4 (0.7) log_{10} IU/ml among patients

Table 2. Baseline viral and host characteristics among genotype-1b ineligible/intolerant patients and their virologic outcome.

Patient	*IL28B* GT	HCV RNA, $\log_{10}$ IU/ml	NS5A polymorphism(s)[a]	NS3 polymorphism(s)[a]	Virologic outcome
P-22	CC	7.1			SVR
P-23	CC	6.9	A92T	Q80L-S122G/S	SVR
P-24	CC	6.6	L28M-R30L-Q54H-A92T	Q80L-S122S/G	D/C at Wk12 due to AE; SVR
P-25	CT	6.8	L31M/L-Y93H/Y		VBT (Wk16)
P-26	CC	5.3			SVR
P-27	CC	6.9	Q54H-Y93H/Y	T54S	SVR
P-28	CC	6.8	Y93H/Y	Q80L	SVR
P-29	CT	6.7	Q54Y-Y93H/Y	Q80L	VBT (Wk16)
P-30	CT	6.7	Q54H		SVR
P-31	CC	6.6	P58S/P-Y93Y/H	S122G	Relapse (FU Wk12)
P-32	CT	6.7	P58L	S122G	Relapse (FU Wk4)
P-33	CT	5.2	Q54H-Q62P/S		D/C at Wk12 due to patient request; SVR
P-34	CC	6.6		Q80L	SVR
P-35	CC	6.4	Q54H-Q62E/A-A92T		SVR
P-36	CC	7.1		S122S/C	Relapse (FU Wk4)
P-37	CC	6.6	Y93H		Relapse (FU Wk4)
P-38	CC	7.5		S122T	SVR
P-39	CC	5.1	R30Q/R		SVR
P-40	CC	6.8	Q54H-A92A/T	Q80L	D/C at Wk8[b]
P-41	CC	6.0		S122G	SVR
P-42	CC	6.5	A92T		SVR
P-43	CT	7.0	Q54Y-Y93H	S122G	VBT (Wk10)

[a]All NS3 and NS5A amino acids were examined with focus on polymorphisms at positions known to be associated with resistance to NS3 protease inhibitors (36, 43, 54, 55, 77, 78, 79, 80, 122, 123, 138, 155, 156, 158, 168, 170, 175) and NS5A inhibitors (21, 23, 24, 28, 30, 31, 32, 54, 58, 62, 92, 93). When a mixture of substitutions is indicated, the most predominant is identified first.
[b]Treatment discontinued at patient request; subsequently lost to follow-up.
AE, adverse event; D/C, discontinued; FU, follow-up; GT, genotype; HCV, hepatitis C virus; SVR, sustained virologic response; VBT, viral breakthrough; Wk, week.

achieving SVR compared with 6.8 (0.3) $\log_{10}$ IU/ml among patients experiencing virologic failure. However, four of six patients with the *IL28B* CT allele subsequently failed treatment (three breakthroughs, one relapse) *vs.* only three of 16 patients with *IL28B* CC (all relapsed).

Genotypic analysis of patients with viral breakthrough.
Treatment-emergent RAVs were assessed through post-treatment week 48 in the three patients with virologic breakthrough (Table 3).

Patient P-25: This patient had an *IL28B* CT genotype with a baseline HCV RNA level of 6.8 $\log_{10}$ IU/ml and a linked baseline NS5A-L31M-Y93H/Y polymorphism. Despite undetectable HCV RNA by week 4 (Fig. 4A), viral breakthrough occurred at week 16, associated with high-level resistance to both DCV (NS5A-L31M-P58A-Y93H; 65,000-fold) and ASV (D168A; ~120-fold in GT1b). Other minor variants detected at baseline by clonal analysis (NS5A-Q62R, -A92T) were not present at breakthrough. NS5A variants present at the end of therapy persisted through follow-up week 48, and, although P58A had largely changed to P58G (73% of 33 clones, Fig. 5A) by week 36, a similar ratio of P58G to A was detected at follow-up week 48. By contrast, NS3-D168A had mostly been replaced by wild type at week 48 (83% of 64 clones).

Patient P-29: This patient had an *IL28B* CT genotype, with a baseline HCV RNA level of 6.7 $\log_{10}$ IU/ml and a pre-existing linked NS5A-Q54Y-Y93H/Y and NS3-Q80L (Fig. 5B). Undetectable HCV RNA by week 3 was followed by viral breakthrough at week 16 (Fig. 4A) associated with NS5A-L31M-Q54Y-Y93H (6467-fold DCV resistance) and NS3-Q80L-D168V (~280-fold ASV resistance). These RAVs remained stable through 48 weeks post-treatment.

Patient P-43: This patient had an *IL28B* CT genotype with a baseline HCV RNA level of 7.0 $\log_{10}$ IU/ml, and a pre-existing NS5A-Q54Y-Y93H variant (Fig. 5C). HCV RNA was undetectable at week 2, and breakthrough occurred at week 10 (Fig. 4A), which was associated with a linked NS5A-L31M-Q54Y-Y93H variant (Fig. 5C; 6467-fold DCV resistance) and an NS3-D168V variant (~270-fold ASV resistance). Again, NS5A variants remained stable through week 48 post-treatment, while NS3-D168V was replaced by wild type (100% of 60 clones).

For the three patients experiencing viral breakthrough, DCV and ASV trough exposures were less than drug levels required to achieve a 90% effective concentration (EC_{90}) value against emergent RAVs (Table 3).

Genotypic analysis of patients experiencing post-treatment relapse.
Four ineligible patients, with undetectable HCV RNA at the end of treatment, experienced relapse (Fig. 4B). Resistance polymorphisms through week 48 off-treatment are shown in Table 3. Baseline polymorphisms associated with resistance were not detected in two patients (P-32 and P-36), but both displayed post-relapse resistance by follow-up weeks 8 and 4, respectively. Patient P-32 relapsed with NS5A-L31M-P58L-Y93H (8300-fold DCV resistance) and NS3-D168V (270-fold ASV resistance).

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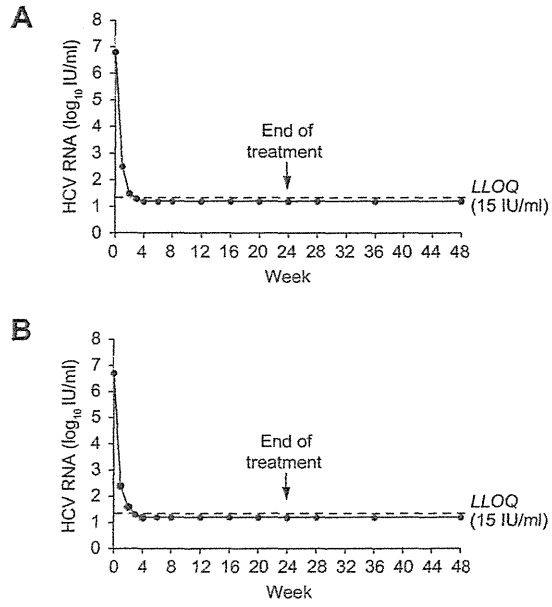

Fig. 1. HCV RNA levels among genotype-1b null responders. Treatment was initiated with (A) asunaprevir 600 mg BID or (B) asunaprevir 200 mg BID, in combination with daclatasvir 60 mg QD. Individual patient HCV RNA levels are shown in grey. Mean HCV RNA levels are shown in black. BID, twice daily; HCV, hepatitis C virus; LLOQ, lower limit of quantitation; QD, once daily.

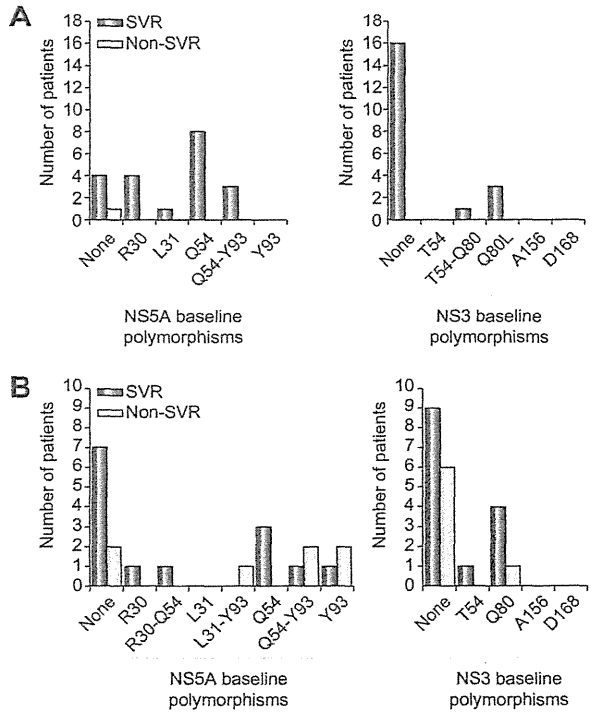

Fig. 2. Impact of baseline polymorphisms associated with resistance on virologic outcome among genotype-1b (A) null responders or (B) ineligible/intolerant patients. The ineligible/intolerant analysis excludes one patient (P-40) who discontinued therapy and was subsequently lost to follow-up. SVR, sustained virologic response.

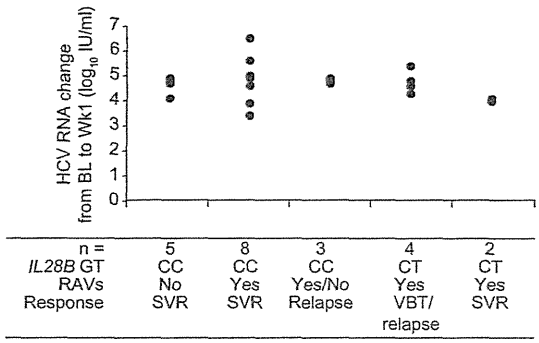

Fig. 3. Early (week 1) declines in HCV RNA were similar among PegIFN-α/RBV ineligible or intolerant patients with and without baseline polymorphisms associated with resistance, virologic failure, and *IL28B* CT genotype. BL, baseline; GT, genotype; HCV, hepatitis C virus; RAV, resistance-associated variant; SVR, sustained virologic response; VBT, viral breakthrough.

Patient P-36 relapsed with an NS5A-L31V/M-Y93H genotype (L31V-Y93H: 14,789-fold DCV resistance *vs.* L31M-Y93H: 7105-fold) [13] and NS3-D168V. The remaining two patients had detectable NS5A-Y93H at baseline (24-fold DCV resistance) and additional substitutions at NS5A-L31 and NS3-D168 were detected after relapse. Patient P-31 displayed NS5A-L31M-Y93H (7105-fold DCV resistance) [13] and NS3-D168A (~120-fold ASV resistance); patient P-37 relapsed with the same NS5A-L31V/M-Y93H and NS3-D168V, as described for patient P-36.

Baseline HCV RNA and *IL28B* genotype did not appear to influence relapse; three of four relapse patients were *IL28B* CC genotype, and baseline HCV RNA was not appreciably higher than for those with SVR (mean HCV RNA [SD]: 6.8 [0.4] *vs.* 6.4 [0.7] $\log_{10}$ IU/ml, respectively).

Changes in the DCV resistance pattern present at relapse through follow-up week 48 were seen in three of four relapsers, with Y93H changing to wild type (100% of 68 clones) in patient P-32. Clonal analysis of the baseline sequence revealed the presence of Y93H as a minor species (~2%; 1/61 clones). Genotypic changes resulting in a lower level of phenotypic resistance (L31V-Y93H to L31M-Y93H) were detected in patients P-36 and P-37. NS3 substitutions observed at relapse were not detectable by population sequencing by follow-up week 36. The D168V substitution detected in patient P-37 was replaced by D168E (78-fold ASV resistance [19]) at follow-up weeks 36 and 48. As with the patients who experienced virologic breakthrough, ASV and DCV trough values in the three drug-compliant patients who relapsed were less than the observed EC_{90} values for the respective RAVs.

Discussion

This study assessed resistance and virologic failure in a difficult-to-treat population of null responders and PegIFN-α/RBV ineligible/intolerant patients treated with the dual oral combination of DCV and ASV. Overall, 77% achieved an SVR [11], with all viral breakthroughs and post-treatment relapses occurring in the ineligible/intolerant subpopulation. It is possible that pharmacokinetics may have played a role in these failures, since patients experiencing failure had DCV and/or ASV trough values below median or documented non-compliance [11]. However, since

Table 3. Emergence of resistance-associated variants among genotype-1b ineligible/intolerant patients experiencing viral breakthrough or relapse.

Patient	Time point	DCV/ASV C_{trough} range, nM	NS5A RAVs				DCV EC_{90}, nM	NS3 RAVs			ASV EC_{90}, nM
			L31	Q54	P58	Y93		Q80	S122	D168	
VBT patients											
P-25	BL		M/L	-	-	H/Y	<137	-	-	-	
	Wk16 (VBT)		M	-	A	H	>1000	-	-	A	540
	Wk20		V	-	A	H		-	-	A	
	Wk24	190-261/25-41	M	-	A	H		-	-	A	
	FU Wk4		M	-	A	H		-	-	A	
	FU Wk36		M	-	G	H	>5000	-	-	D/A	
	FU Wk48		M	-	G/A	H		-	-	-	
P-29	BL		-	Y	-	H	0.04	L	-	-	1.6
	Wk16 (VBT)		ND	ND	ND	ND	ND	ND	ND	ND	ND
	Wk20	116-198/18-33	M/V	Y	-	H	750	L	-	V	55
	FU Wk4		M	Y	-	H		L	-	V	
	FU Wk36		M	Y	-	H		L	-	V	
	FU Wk48		M	Y	-	H		L	-	V	
P-43	BL		-	Y	-	H	0.49	-	G	-	2.8
	Wk10 (VBT)		M	Y	-	H	435	-	G	V	279
	FU Wk4	243/69	M	Y	-	H		-	G	V	
	FU Wk36		M	Y	-	H		-	G	-	
	FU Wk48		M	Y	-	H		-	G	-	
Relapse patients											
P-31	BL	573-620/ 153-327	-	-	S/P	Y/H	0.02	-	G	-	
	FU Wk16		ND					-	-	A	
	FU Wk24		M	-	-	H	351	-	G	-	
	FU Wk36		-	-	-	-		-	-	-	
	FU Wk48		-	-	-	-		-	-	-	
P-32	BL	151-306/19-42	-	-	L	-	0.004	-	G	-	
	FU Wk8		M	-	L	H		-	G	V/D	
	FU Wk12		M	-	L	H	543	-	G	-	
	FU Wk36		M	-	L	-	1.5	-	G	-	
	FU Wk48		M	-	L	-		-	G	-	
P-36	BL	138/26	-	-	-	-		-	-	-	
	FU Wk8		V/M	-	-	H		-	-	V	1190
	FU Wk12		V	-	-	H	349	-	-	-	
	FU Wk24		M/V	-	-	H		-	-	V/D	
	FU Wk36		M	-	-	H	137	-	-	-	
	FU Wk48		M	-	-	H		-	-	-	
P-37	BL	75-134/40-93	-	-	-	H	0.49	-	-	-	
	FU Wk8		V	-	-	H		-	-	V	
	FU Wk12		V/I	-	-	H		-	-	V	
	FU Wk24		M	-	-	H		-	-	V	
	FU Wk36		M	-	-	H		-	-	E/D	
	FU Wk48		M	-	-	H		-	-	-	

When a mixture of substitutions is indicated, the most predominant is written first. ASV-resistant variants conferred no cross-resistance to DCV and *vice versa* in a replicon assay. Dashes indicate consensus with control sequence GT1b (Con1).

ASV, asunaprevir; BL, baseline; DCV, daclatasvir; EC_{90}, 90% effective concentration; FU, follow-up; ND, not determined as multiple amplifications failed; RAV, resistance-associated variant; VBT, viral breakthrough; Wk, week.

most patients with troughs below the median achieved SVR, the influence of drug exposure is hard to assess.

NS5A-Y93H was identified as the predominant polymorphism at baseline in all three patients with viral breakthrough and in two of the four patients with relapse. However, three null responders and two ineligible/intolerant patients also had a pre-existing NS5A-Y93H polymorphism and all achieved SVR, making the significance of Y93H alone, for response in the broader patient population, difficult to assess. Furthermore, where Y93H polymorphisms existed at baseline, their effects on

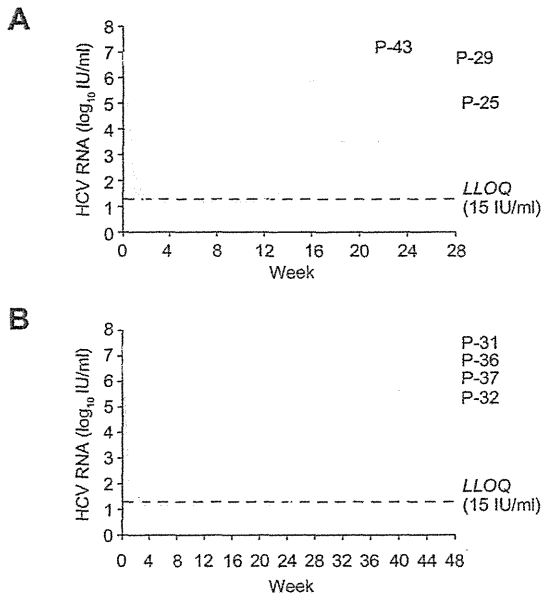

Fig. 4. HCV RNA levels on-treatment and during post-treatment follow-up for genotype-1b ineligible/intolerant patients experiencing (A) viral breakthrough or (B) relapse. Solid lines indicate on-treatment period. Dashed lines indicate post-treatment follow-up. HCV, hepatitis C virus; LLOQ, lower limit of quantitation.

DCV inhibition were minimal (Y93H EC_{50} = 49 pM [6]) compared with C_{trough} values that ranged from 75 to 620 nM. The global prevalence of NS5A-Y93H is approximately 4%, based on data from the Los Alamos database [20] and unpublished data from nine DCV studies, and is approximately 11% in other recent Japanese DCV studies [21], which is considerably lower than the 23% (10/43) prevalence observed in this study. Further analysis of DCV study data indicates that Y93H pre-exists at higher levels in patients infected with GT1b (10%) than GT1a (1%); however, the link with *IL28B* is not so clear given that most failures to date with DCV have been observed in GT1a patients with no baseline Y93H. Other polymorphisms observed at a higher frequency among this GT1b population included NS3-Q80L (~19%, 8/43) *vs.* Q80K, which has been observed more frequently in GT1a populations [18,19].

Baseline HCV RNA did not appear to influence virologic response in either population, and response was too rapid to allow successful genomic sequencing after 1 week of treatment. ASV dose (600 mg or 200 mg twice daily) did not impact the initial decline in HCV RNA in null responders, and the *IL28B* CT allele, present in 86% (18/21) of null responders, did not prevent patients from achieving a very high (90%) SVR. By contrast, although only 27% (6/22) of ineligible/intolerant patients were *IL28B* CT, this genotype was present in all three viral breakthroughs and one of four relapses. While *IL28B* genotype is known to influence response to PegIFN-α/RBV, its apparent impact on virologic suppression in alfa-sparing regimens is unexpected. However, given the small number of patients, any such correlation will require evaluation in a larger dataset.

The emergent RAVs at viral breakthrough or relapse (signature NS5A-L31 and -Y93 substitutions for DCV and NS3-D168 substitutions for ASV) were similar to observations from other

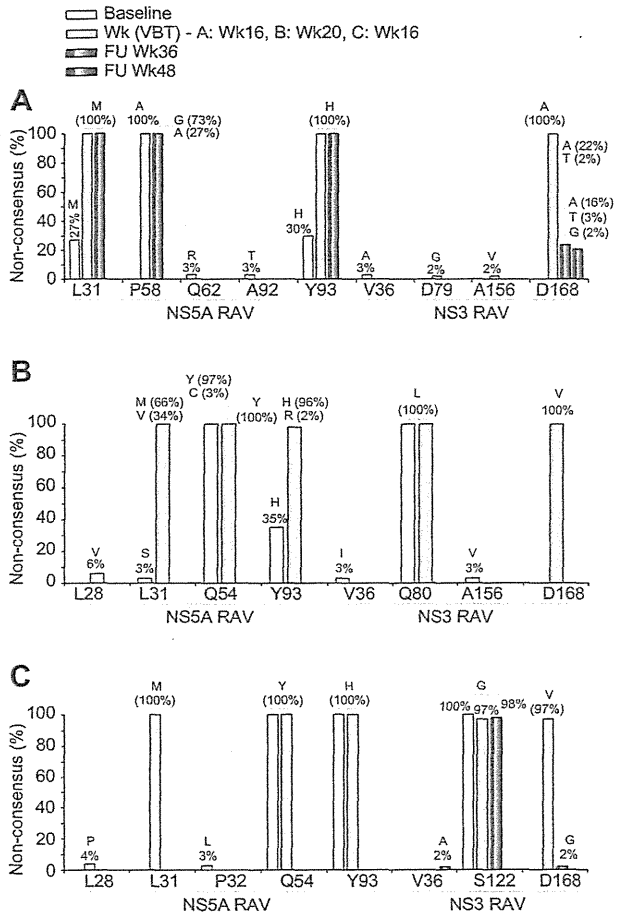

Fig. 5. Clonal analysis of NS3 protease and NS5A resistance-associated variants in patients experiencing virologic breakthrough. (A) Patient P-25. *NS5A RAV: baseline 30 clones; Wk16 39 clones; FU Wk36 33 clones; FU Wk48 not* performed (no change from FU Wk36 by population sequencing). NS3 RAV: baseline 32 clones; Wk16 41 clones; FU Wk36 56 clones; FU Wk48 63 clones. (B) Patient P-29. NS5A RAV: baseline 37 clones; Wk20 50 clones; FU Wk36/48 analyses not performed (no change from VBT by population sequencing). NS3 RAV: baseline 34 clones; Wk20 47 clones; FU Wk36/48 analyses not performed (no change from VBT by population sequencing). (C) Patient P-43. NS5A RAV: baseline 32 clones; Wk10 47 clones; FU Wk36/48 analyses not performed (no change from VBT by population sequencing). NS3 RAV: baseline 31 clones; Wk10 32 clones; FU Wk36 103 clones; FU Wk48 60 clones. FU, follow-up; VBT, viral breakthrough; RAV, resistance-associated variant.

clinical studies of DCV, and from *in vitro* GT1b replicon resistance studies with ASV [19], although this study represents the first demonstration of emergent clinical ASV resistance. It is possible that signature resistance variants to both DCV and ASV pre-existed as minor species, and subsequently enriched by selective pressure, as predicted by viral kinetic modeling [22]. Although a combination of these NS3 and NS5A variants was not detected by clonal sequencing at baseline, their low-level pre-existence cannot be ruled out. However, assessment of minor NS3 plus NS5A variants from the same RNA sequence is currently not feasible using available deep-sequencing technologies. Nevertheless, additional studies to assess the presence and dynamics of minority baseline variants under drug selection are indicated.

Interestingly, ASV-resistant NS3-D168 substitutions generally decayed during the off-drug follow-up period, implying a lack of replicative fitness relative to wild type, in the absence of selective drug pressure. Indeed, a reduction in replicative fitness has been observed for D168 variants in replicons [19]. Neither of the secondary variants associated with D168V in this study (Q80L or S122G) had an impact on fitness *in vitro* (replication capacity similar or higher than that observed for parental GT1b [Con1] replicon), with both double variants possessing replicative capacities similar to D168V alone [19]. However, clonal analysis indicated that ASV-resistant variants were still detectable in some post-treatment samples as minority species, although not detectable by population sequencing. Deeper sequencing techniques will be required to fully establish the dynamics of decay and whether ASV-resistant strains remain enriched for long periods relative to baseline. Since the re-treatment of patients with prior NS3 protease inhibitor failure has only been assessed in small studies [23], it is not clear whether these NS3 RAVs will form a stable minority capable of rapid overgrowth on re-treatment. By contrast, NS5A variants associated with DCV resistance were observed to be linked and relatively stable through at least 48 weeks post-treatment, although change of DCV-resistance substitutions was noted in four of seven patient samples. As described above, the prevalence of the NS5A variant Y93H, which confers low level resistance to DCV, is approximately 10% in the general HCV GT1b population. Linked NS5A RAVs conferring high level resistance to DCV are less prevalent (<1%). While NS3 RAVs (substitutions at positions V36, T54, R155, or D168) associated with first-generation protease inhibitors have been reported to be present at ⩽2.7% by population sequencing [5,24], emergent NS3 RAVs have been shown to persist for up to 4 years in long-term follow-up studies [25]. Therefore, longer-term studies are indicated to assess what, if any, replicative impairment is conferred by these linked NS5A changes and how long these potentially transmissible drug-resistant strains persist without DCV selection pressure.

In conclusion, high response rates were achieved in this small Japanese study comprising GT1b null responders and PegIFN-α/RBV ineligible/intolerant patients with limited treatment options. Among patients experiencing virologic failure, ASV- and DCV-resistant substitutions emerged together at the time of failure, which were similar to those reported previously. An analysis of persistence demonstrated that DCV-resistant substitutions appeared to have greater fitness over the duration of the study. A loose association with a baseline NS5A polymorphism on virologic outcome was observed; however, further data from larger studies are required. Consequently, a greater understanding of the role and dynamics of pre-existing, emergent, and persistent resistance variants to DCV and ASV will be sought from the planned phase 3 global studies of this combination.

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Conflict of interest

K Chayama has received research grants and consulting fees from Bristol-Myers Squibb, Dainippon Sumitomo Pharma, Mitsubishi Tanabe Pharma, Daiichi Sankyo, Toray Industries, Otsuka Pharmaceutical Company, and GlaxoSmithKline KK. Hiroki Ishikawa, Hideaki Watanabe, Wenhua Hu, Dennis Hernandez, Fei Yu, and Fiona McPhee are employees of Bristol-Myers Squibb. All other authors have no conflicts to report.

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Original Article

Randomized controlled trial of a new procedure of radiofrequency ablation using an expandable needle for hepatocellular carcinoma

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Aim: To evaluate the efficacy of a new ablation procedure for the stepwise hook extension technique using a SuperSlim needle for radiofrequency ablation (RFA) treatment of hepatocellular carcinoma (HCC), a randomized controlled trial was performed.

Methods: Thirty patients with HCC measuring 20 mm or less were randomly treated with a conventional four stepwise expansion technique (group 1) and the new stepwise expansion technique (group 2; the electrode was closed in the shaft after the same three steps of the conventional procedure and then fully extended). All patients underwent the RFA procedure using a 10-hook expandable electrode of 17-G diameter (LeVeen SuperSlim 30 mm). We compared the ablation time, required energy and ablated lesions in the two groups.

Results: The long and short diameters of RFA-induced necrosis were significantly larger in group 2 (37 and 28 mm) than group 1 (30 and 26 mm, $P = 0.001$ and $=0.045$, respectively). Irregular and small needle expansion resulting in the parachute-like or irregularly shaped ablated zone was observed in more cases in group 1 than in group 2. The new technique made all tines expand uniformly and largely, which produced a near-oval ablated zone of which the long axis is perpendicular to the needle shaft.

Conclusion: The two kinds of stepwise procedures allow the selection of a more suitable procedure according to the tumor size and shape in each RFA.

Key words: expandable needle, hepatocellular carcinoma, radiofrequency ablation, randomized controlled trial

INTRODUCTION

PERCUTANEOUS TREATMENT INCLUDING radiofrequency ablation (RFA) and percutaneous ethanol injection (PEI) is often used for small-size hepatocellular carcinoma (HCC) because it is less invasive than surgical therapy. RFA has become the first-choice local treatment because of the excellent outcome; the efficacy of RFA in HCC tumors measuring less than 2 cm in diameter is similar to that of PEI but it requires fewer treatment sessions, and the efficacy in HCC tumors of more than 2 cm in diameter is better than with PEI.[1] In addition, RFA is also more cost-effective than surgical resection of small HCC.[2] With three commercially-available RFA apparatuses – the radiofrequency tumor coagulation system (RTC system; Boston-Scientific, Natick, MA, USA), radiofrequency interstitial thermal ablation system (RITA; AngioDynamics, Latham, NY, USA) and cool-tip RF system (Valleylab, a division of Tyco Healthcare Group, Boulder, CO, USA) – the volume ablated during one RFA session is of a diameter less than 3.0–4.0 cm, except in ablation with the Starburst XL RFA device (RITA).[3] RFA therapy is currently restricted to tumors measuring less than 3 cm. In this regard, previous studies reported that the necrotic area could be enlarged by saline injection prior to RFA,[4,5] combination of RFA with PEI,[6,7] RFA with ethanol lipiodol injection,[8] RFA with transcatheter arterial embolization[9] and RFA with transient arterial obliteration.[10–12]

Among the above three RFA apparatuses, the RTC system and RITA have adopted the use of expandable needles. We reported previously the efficacy of the

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stepwise hook extension technique for RFA therapy of HCC.[13] The technique allows rapid roll-off at lower power and lower energy and reduces any possible increase in intra-tissue pressure that may cause scattering of intrahepatic metastasis.[14–17]

A more slender expandable needle has been developed (17-G, SuperSlim; Boston Scientific, Natick, MA, USA) for easier and safer insertion into the liver. However, insertion of the slim needle into the liver tissue could result in deformation of the needle and hence possible reduction of the size of the ablated area. To overcome this shortfall, we designed a new technique involving full re-expansion after stepwise extension, to ensure full expansion of the needle. We have already reported the experimental study using healthy pig livers *in vivo* to show that this technique can produce a larger necrotic zone than the conventional stepwise procedure.[18]

The aim of this study was to evaluate the efficacy of the new ablation procedure for the stepwise hook extension technique for RFA therapy of HCC of a patient with cirrhosis or without cirrhosis in a randomized controlled trial.

METHODS

Patients and tumors

FROM NOVEMBER 2006 to March 2010, 30 consecutive patients who met the following criteria were enrolled in this study: (i) HCC confirmed either histopathologically or radiologically; and (ii) diameter of the hepatic tumor of no more than 20 mm. They included 20 men and 10 women, with a median age of 57 years (range, 43–73). Seventeen patients were with cirrhosis and the other 13 were without cirrhosis. Table 1 lists the clinical background of patients of both groups. There were no significant differences between the groups.

A typical hypervascular HCC was diagnosed by typical hypervascular stain on digital subtraction angiography. In addition, one of the following three criteria was used to diagnose a tumor as a well-differentiated HCC: (i) histopathological diagnosis as well-differentiated HCC; (ii) hypo-enhanced lesion on computed tomography (CT) during hepatic arteriography (CTHA) and hypoperfusion on CT during arterial portography (CTAP); and (iii) hypo-enhanced lesion on the equilibrium phase of dynamic CT or hypo-perfused lesion on CTAP and hypointense on the hepatocyte-specific phase of multiple resonance imaging (MRI) using gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid (Gd-EOB-DTPA) (Primovist; Bayer Schering Pharma, Osaka, Japan). A total of 30 patients were treated by the RFA protocol.

The study protocol was approved by the Human Ethics Review Committee of Toranomon Hospital and a signed consent form was obtained from each patient.

RFA protocol

We used the RTC system comprising a slim expandable needle (30 mm, 17-G LeVeen needle, SuperSlim), which consists of 10 expandable monopolar array electrodes, and the RF3000 generator, with a maximum power output of 120 W, and four electrode pads placed on the patient's skin. Instead of using the standard method recommended by the manufacturer, we adopted two types of stepwise hook extension techniques.[18] Patients were randomly divided into two groups based on the RFA protocol used. In group 1, after placing the needle electrode shaft into the tumor with the array retracted, using real-time ultrasound guidance, the electrode tines were expanded to a quarter, a half, three-quarters of the length and full-length in the first, second, third and final steps, respectively. The diameter of the array at each step was 10, 15, 25 and 30 mm, respectively. In group 2, the

Table 1 Background of the patients in groups 1 and 2

	Group 1 (conventional method)	Group 2 (new method)	*P*
Male : female	8:8	12:2	0.042
Age†	69 (45–82)	71 (60–84)	0.270
With cirrhosis: without cirrhosis	11:5	6:8	0.160
Tumor diameter, mm†	12 (9–18)	16 (6–19)	0.179
Hypervascular, yes : no	13:3	11:3	0.520

†Data are median (range).
NS, not significant.