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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DOI 10.1002/hep.26087

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

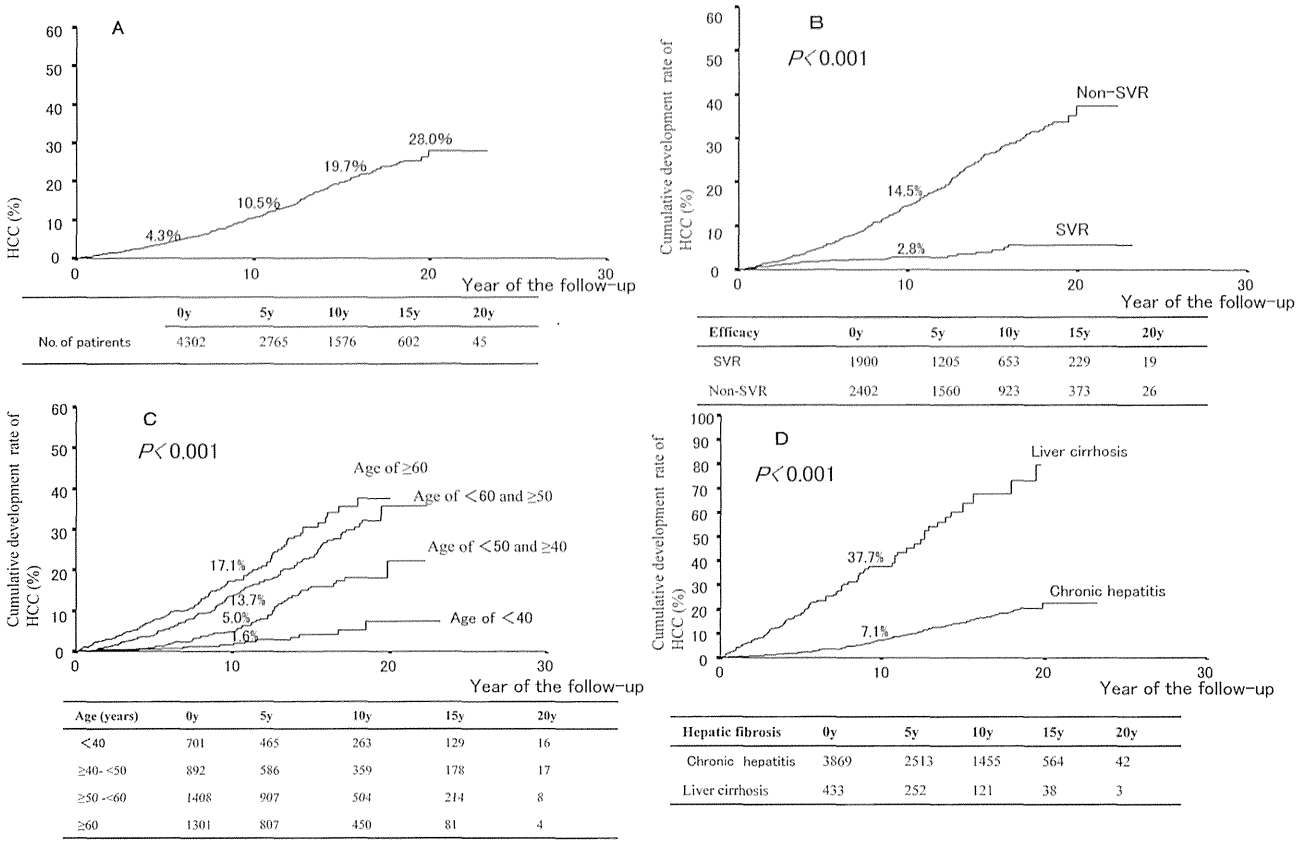


Fig. 1. Cumulative development rate of HCC (A) in total HCV patients treated with IFN therapy and based on the difference of (B) efficacy, (C) age, and (D) hepatic fibrosis.

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

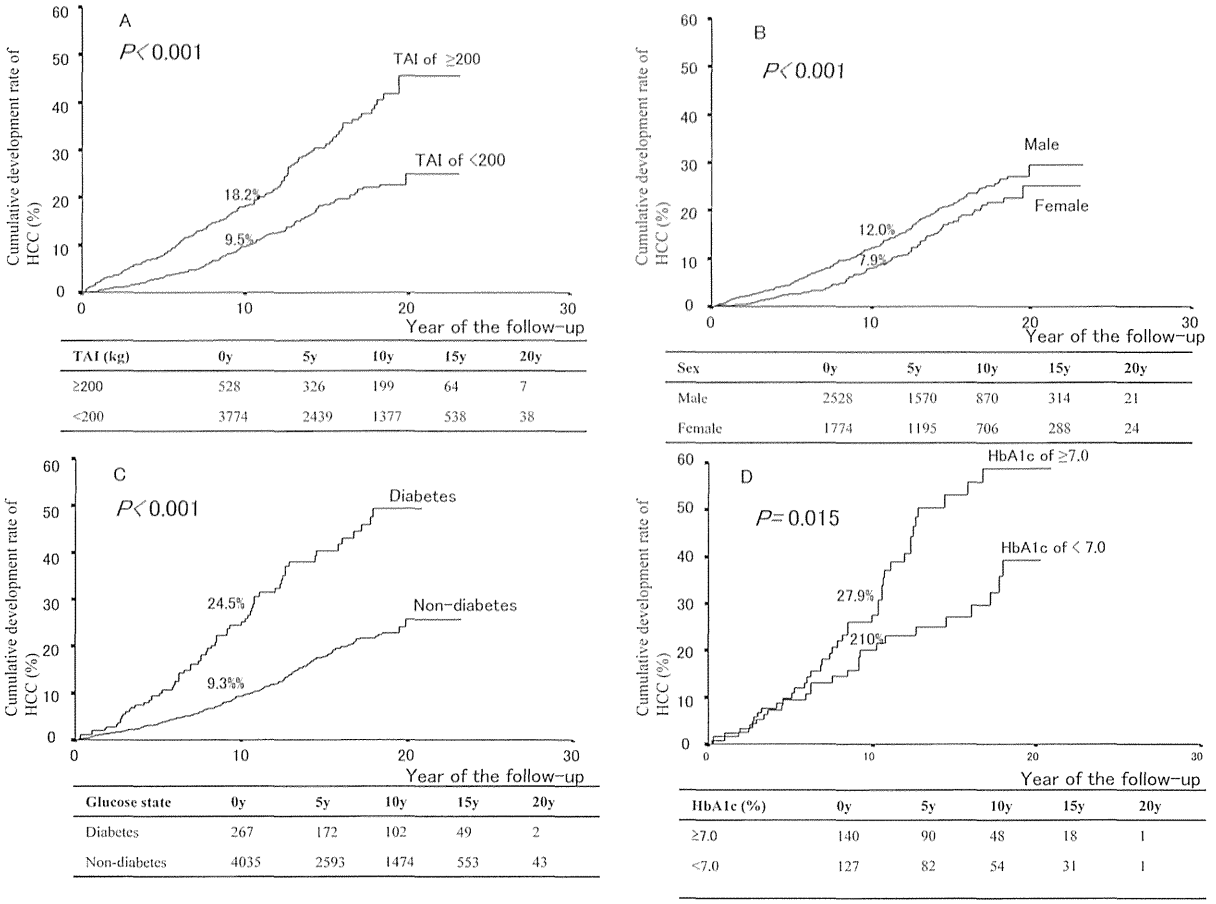


Fig. 2. Cumulative development rate of HCC based on the difference of (A) TAI, (B) sex, (C) diabetic state, and (D) mean HbA1c level during follow-up in T2DM patients.

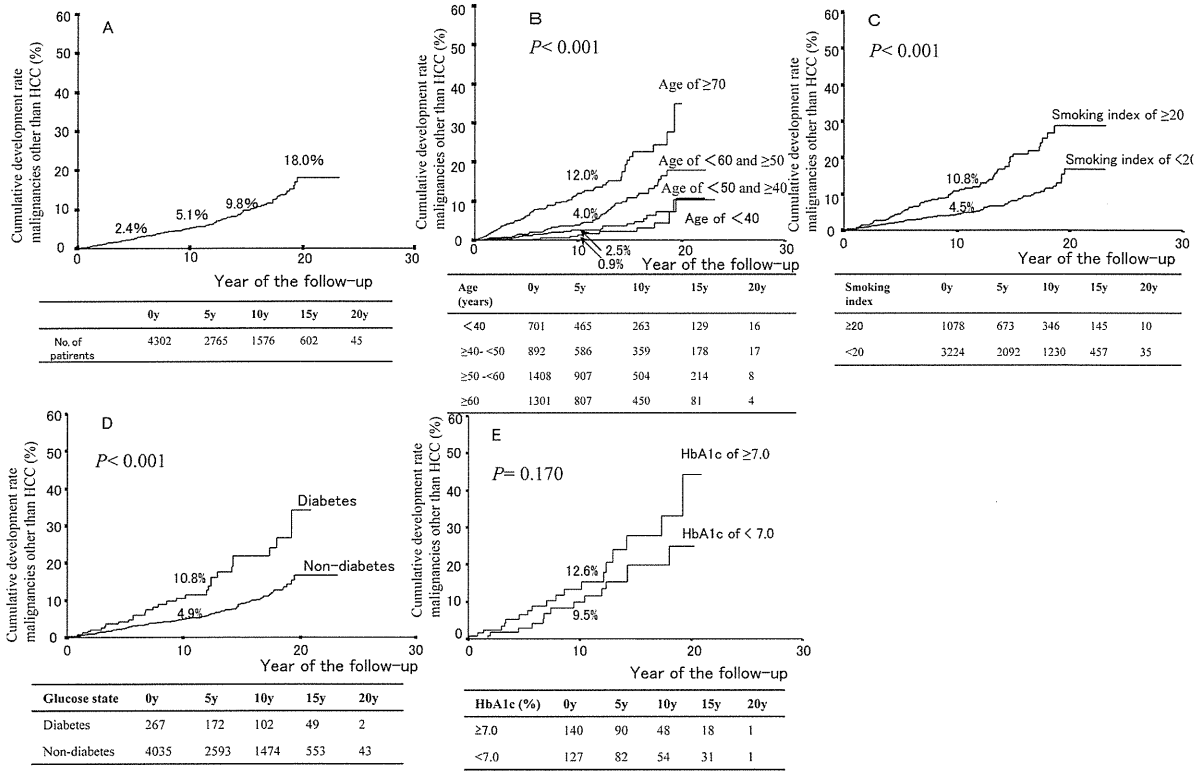
patients with mean HbA1c level of <7.0% versus those with mean HbA1c level of ≥7.0% was 0.62 (95% CI, 0.31-1.23; $P = 0.170$). There was no significant difference in development of malignancies other than HCC based on the difference of mean HbA1c level during follow-up. Table 5 shows the impact based

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.



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

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

Smoking index	0y	5y	10y	15y	20y
≥20	1078	673	346	145	10
<20	3224	2092	1230	457	35

Glucose state	0y	5y	10y	15y	20y
Diabetes	267	172	102	49	2
Non-diabetes	4035	2593	1474	553	43

HbA1c (%)	0y	5y	10y	15y	20y
≥7.0	140	90	48	18	1
<7.0	127	82	54	31	1

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.

Acknowledgment: We thanks Thomas Hughes for editorial assistance.

References

1. Kiyosawa K, Furuta S. Review of hepatitis C in Japan. J Gastroenterol Hepatol 1991;6:383-391.
2. Alter MJ, Margolis HS, Krawczynski K, Judson FN, Mares A, Alexander WJ, et al. The natural history of community acquired hepatitis C in the United States. N Engl J Med 1992;327:1899-1905.
3. Colombo M, Kuo G, Choo QL, Donato MF, Del Ninno E, Tommasini MA, et al. Prevalence of antibodies to hepatitis C virus in Italian patients with hepatocellular carcinoma. Lancet 1989;2:1006-1008.
4. Hasan F, Jeffers LJ, De Medina M, Reddy KR, Parker T, Schiff ER, et al. Hepatitis C-associated hepatocellular carcinoma. HEPATOLOGY 1990;12:589-591.
5. Kew MC, Houghton M, Choo QL, Kuo G. Hepatitis C virus antibodies in southern African blacks with hepatocellular carcinoma. Lancet 1990;335:873-874.
6. Tsukuma H, Hiyama T, Tanaka S, Nakao M, Yabuuchi T, Kitamura T, et al. Risk factors for hepatocellular carcinoma among patients with chronic liver disease. N Engl J Med 1993;328:1797-1801.
7. Ikeda K, Saitoh S, Koida I, Arase Y, Tsubota A, Chayama K, et al. A multivariate analysis of risk factors for hepatocellular carcinogenesis: a prospective observation of 795 patients with viral and alcoholic cirrhosis. HEPATOLOGY 1993;18:47-53.
8. Waki K, Noda M, Sasaki S, Matsumura Y, Takahashi Y, Isogawa A, et al.; JPHC Study Group. Alcohol consumption and other risk factors for self-reported diabetes among middle-aged Japanese: a population-based prospective study in the JPHC study cohort I. Diabet Med 2005;22:323-331.
9. Yasuda K. Early gastric cancer: diagnosis, treatment techniques and outcomes. Eur J Gastroenterol Hepatol 2006;18:839-845.
10. Van Gossum A. Guidelines for colorectal cancer screening—a puzzle of tests and strategies. Acta Clin Belg 2010;65:433-436.
11. Currie GP, Kennedy AM, Denison AR. Tools used in the diagnosis and staging of lung cancer: what's old and what's new? QJM 2009;102:443-448.
12. Maresh EL, Mah V, Alavi M, Horvath S, Bagryanova L, Liebeskind ES, et al. Differential expression of anterior gradient gene AGR2 in prostate cancer. BMC Cancer 2010;10:680.
13. Calle EE, Rodriquez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. N Eng J Med 2003;348:1625-1638.
14. Harris NL, Jaffe ES, Stein H, Banks PM, Chan JK, Cleary ML, et al. A revised European-American classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group. Blood 1994;84:1361-1392.
15. Cascinu S, Falconi M, Valentini V, Jelic S; ESMO Guidelines Working Group. Pancreatic cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 2010;21(Suppl. 5):v55-v58.
16. American Diabetes Association. Diagnosis and classification of diabetes mellitus. Diabetes Care 2010;33(Suppl. l):S62-S69.

17. Dusheiko G, Schmilovitz-Weiss H, Brown D, McOmish F, Yap PL, Sherlock S, et al. Hepatitis C virus genotypes: an investigation of type-specific differences in geographic origin and disease. HEPATOLOGY 1994; 19:13-18.
18. Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Sheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. HEPATOLOGY 1994;19:1513-1520.
19. Fleming TR, Harrington DP, O'Brien PC. Designs for group sequential tests. Control Clin Trials 1984;5:348-361.
20. Harrington DP, Fleming TR. A class of rank test procedures for censored survival data. Biomedaica 1983;62:205-209.
21. Kaplan EL, Meier P. Nonparametric estimation for incomplete observation. J Am Stat Assoc 1958;53:457-481.
22. Cox DR. Regression models and life tables. J R Stat Soc 1972;34: 248-275.
23. Ikeda K, Saitoh S, Arase Y, K Chayama, Y Suzuki, M Kobayashi, et al. Effect of interferon therapy on hepatocellular carcinogenesis in patients with chronic hepatitis type C; A long-term observation study of 1643 patients using statistical bias correction with proportional hazard analysis. HEPATOLOGY 1999;29:1124-1130.
24. Kasahara A, Hayashi N, Mochizuki K, Takayanagi M, Yoshioka K, Kakumu S, et al. Risk factors for hepatocellular carcinoma and its incidence after interferon treatment in patients with chronic hepatitis C. Osaka Liver Disease Study Group. HEPATOLOGY 1998:2;1394-1402.
25. Imai Y, Kawata S, Tamura S, Yabuuchi I, Noda S, Inada M, et al. Relation of interferon therapy and hepatocellular carcinoma in patients with chronic hepatitis C. Osaka Hepatocellular Carcinoma Prevention Study Group. Ann Intern Med 1998:129;94-99.
26. Yoshida H, Arakawa Y, Sata M, Nishiguchi S, Ya M, Fujiyama S, et al. Interferon therapy prolonged life expectancy among chronic hepatitis patients. Gastroenterology 2002:123;483-491.
27. Veldt BJ, Chen W, Heathcote EJ, Wedemeyer H, Reichen J, Hofmann WP, et al. Increased risk of hepatocellular carcinoma among patients with hepatitis C cirrhosis and diabetes mellitus. HEPATOLOGY 2008;47: 1856-1862.
28. Asahina Y, Tsuchiya K, Tamaki N, Hirayama I, Tanaka T, Sato M, et al. Effect of aging on risk for hepatocellular carcinoma in chronic hepatitis C virus infection. HEPATOLOGY 2010;52:518-527.
29. Matsuda T, Marugame T, Kamo KI, Katanoda K, Ajiki W, Sobue T. The Japan Cancer Surveillance Research Group. Cancer incidence and incidence rates in Japan in 2005: based on data from 12 population-based cancer registries in the Monitoring of Cancer Incidence in Japan (MCIJ) Project. Jpn J Clin Oncol 2011;41:139-147.
30. Boyle P. Cancer, cigarette smoking and premature death in Europe: a review including the Recommendations of European Cancer Experts Consensus Meeting, Helsinki, October 1996. Lung Cancer 1997;17:1-60.
31. Botteri E, Iodice S, Bagnardi V, Raimondi S, Lowenfels AB, Maisonneuve P. Smoking and colorectal cancer: a meta-analysis. JAMA 2008; 300:2765-2778.
32. Everhart J, Wright D. Diabetes mellitus as a risk factor for pancreatic cancer. A metaanalysis. JAMA 1995;273:1605-1609.
33. El-Serag HB, Hampel H, Javadi F. The association between diabetes and hepatocellular carcinoma: a systematic review of epidemiologic evidence. Clin Gastroenterol Hepatol 2006;4:369-380.
34. Larsson SC, Orsini N, Brismar K, Wolk A. Diabetes mellitus and risk of bladder cancer: a meta-analysis. Diabetologia 2006;49:2819-2823.
35. Mitri J, Castillo J, Pittas AG. Diabetes and risk of non-Hodgkin's lymphoma: a metaanalysis of observational studies. Diabetes Care 2008;31: 2391-2397.
36. Larsson SC, Orsini N, Wolk A. Diabetes mellitus and risk of colorectal cancer: a metaanalysis. J Natl Cancer Inst 2005;97:1679-1687.
37. Giovannucci E. Metabolic syndrome, hyperinsulinemia, and colon cancer: a review. Am J Clin Nutr 2007;86:836S-842S.
38. Larsson SC, Mantzoros CS, Wolk A. Diabetes mellitus and risk of breast cancer: a metaanalysis. Int J Cancer 2007;121:856-862.
39. Hsing AW, Gao Y-T, Chua S, Deng J, Stanczyk FZ. Insulin resistance and prostate cancer risk. J Natl Cancer Inst 2003;95:67-71.
40. George AJ, Thomas WG, Hannan RD. The renin-angiotensin system and cancer: old dog, new tricks. Nat Rev Cancer 2010;10:745-759.
41. Shackelford DB, Shaw RJ. The LKB1-AMPK pathway: metabolism and growth control in tumour suppression. Nat Rev Cancer 2009;9: 563-575.
42. Brown MS, Goldstein JL. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. Cell 1997;89:331-340.
43. Mehta SH, Brancati FL, Strathdee SA, Pankow JS, Netski D, Coresh J, et al. Hepatitis C virus infection and incident type 2 diabetes. HEPATOLOGY 2003;38:50-56.
44. Imazeki F, Yokosuka O, Fukai K, Kanda T, Kojima H, Saisho H. Prevalence of diabetes mellitus and insulin resistance in patients with chronic hepatitis C: comparison with hepatitis B virus-infected and hepatitis C virus-cleared patients. Liver Int 2008;28:355-362.
45. Arase Y, Suzuki F, Suzuki Y, Akuta N, Kobayashi M, Kawamura Y, et al. Sustained virological response reduces incidence of onset of type 2 diabetes in chronic hepatitis C. HEPATOLOGY 2009;49:739-744.
46. Thuluvath PJ, John PR. Association between hepatitis C, diabetes mellitus, and race. a case-control study. Am J Gastroenterol 2003;98: 438-441.

HEPATOLOGY
Official Journal of the American Association for the Study of Liver Diseases

Complicated Relationships of Amino Acid Substitution in Hepatitis C Virus Core Region and *IL28B* Genotype Influencing Hepatocarcinogenesis

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The impact of amino acid (aa) 70 substitution in the core region on hepatocarcinogenesis and survival for liver-related death in patients of hepatitis C virus (HCV) genotype 1b (HCV-1b), who had not received antiviral therapy, is unknown. The relationships among aa 70 substitution, *IL28B* genotype, and hepatocarcinogenesis are also not clear. A total of 1,181 consecutive HCV-infected patients, who had not received antiviral therapy, were included in a follow-up study to determine predictive factors of hepatocarcinogenesis and survival for liver-related death. The cumulative hepatocarcinogenesis rates in HCV-1b of Gln70(His70) (glutamine (histidine) at aa 70) were significantly higher than those in HCV-1b of Arg70 (arginine at aa 70) and HCV-2a/2b. The cumulative survival rates for liver-related death in HCV-1b of Gln70(His70) were significantly lower than those in HCV-1b of Arg70 and HCV-2a/2b. Multivariate analysis identified gender (male), age (≥60 years), albumin (<3.9 g/dL), platelet count (<15.0 × 10^4/mm^3), aspartate aminotransferase (≥67 IU/L), and HCV subgroup (HCV-1b of Gln70(His70)) as determinants of both hepatocarcinogenesis and survival rates for liver-related death. In HCV-1b patients, the cumulative change rates from Arg70 to Gln70(His70) by direct sequencing were significantly higher than those from Gln70(His70) to Arg70. In patients of Arg70 at the initial visit, the cumulative change rates from Arg70 to Gln70(His70) in *IL28B* rs8099917 non-TT genotype were significantly higher than those in the TT genotype. *Conclusion:* Substitution of aa 70 in the core region of HCV-1b is an important predictor of hepatocarcinogenesis and survival for liver-related death in HCV patients who had not received antiviral therapy. The *IL28B* genotype might partly affect changes over time of dominant amino acid in core aa 70 of HCV-1b. (HEPATOLOGY 2012;56:2134-2141)

Hepatitis C virus (HCV) usually causes chronic infection that can result in chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC).[1,2] At present, treatments based on interferon (IFN), in combination with ribavirin, are the mainstay for combating HCV infection. In Japan, HCV genotype 1b (HCV-1b) and high viral loads account for more than 70% of HCV infections, making it difficult to treat patients with chronic hepatitis C.[3]

Despite numerous lines of epidemiologic evidence connecting HCV infection and the development of HCC, it remains controversial whether HCV itself plays a direct role or an indirect role in the pathogenesis of HCC.[4] It has become evident that HCV core region has oncogenic potential through the use of transgenic mice, but the clinical impact of the core region on hepatocarcinogenesis is still unclear.[5] Previous reports indicated that amino acid (aa) substitutions at position 70 in the HCV core region of patients infected with HCV-1b are pretreatment predictors of poor virological response to pegylated IFN (PEG-IFN)/ribavirin combination therapy and triple therapy

Abbreviations: aa, amino acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; PEG/IFN, pegylated interferon.

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Received May 1, 2012; Revised May 30, 2012; accepted June 17, 2012.

Supported in part by Grants-in-Aid for scientific research and development from the Ministry of Health, Labor and Welfare and Ministry of Education Culture Sports Science and Technology, Government of Japan.

of telaprevir/PEG-IFN/ribavirin,[6-9] and also affects hepatocarcinogenesis.[10-13] These reports support the findings of oncogenic potential by core region from the clinical aspect. However, its impact on hepatocarcinogenesis and survival for liver-related death in patients of HCV-1b who had not received antiviral therapy is still unknown.

The *IL28B* genotype is a poor predictor of virological response to PEG-IFN/ribavirin combination therapy and triple therapy of telaprevir/PEG-IFN/ribavirin[9,14-17] and is reported to be associated with HCC, although its impact on HCC is controversial.[18-21] Furthermore, treatment-resistant substitution of core aa 70 (glutamine/histidine at aa 70 (Gln70/His70)), which might affect hepatocarcinogenesis, was significantly more frequent in patients with treatment-resistant genotype (non-TT) than -sensitive genotype (TT) at *IL28B* rs8099917.[21-23] Thus, the significant linkage between substitution of aa 70 and *IL28B* genotype had been shown, but it is not clarified whether the existence of a complex interaction between the virus and host might affect hepatocarcinogenesis.

The present study included 1,181 consecutive HCV-infected patients who had not received antiviral therapy. The aims of the study were: (1) To evaluate the impact of aa substitutions in the core region of HCV-1b on hepatocarcinogenesis and survival for liver-related death; and (2) To investigate the association of *IL28B* genotype and time-dependent aa changes in the core region of HCV-1b.

Patients and Methods

Patients. Among 2,799 consecutive HCV-infected patients in whom antiviral therapy (IFN monotherapy or IFN plus ribavirin combination therapy) was not induced between December 1962 and November 2010 at Toranomon Hospital, 1,181 were selected in the present study based on the following criteria. (1) Positive for anti-HCV (third-generation enzyme immunoassay, Chiron, Emerville, CA) and positive for HCV RNA (nested polymerase chain reaction [PCR]), at the initial visit. (2) Patients without HCC at the initial visit. (3) Patients infected with single genotype of HCV-1b, 2a, or 2b. (4) In HCV-1b, patients analyzed aa substitutions of the core region by direct sequencing, one or more times from the initial visit. (5) Patients negative for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo, Japan). (6) Patients free of coinfection with human immunodeficiency virus. (7) Patients free of other types of chronic liver disease, including hemochromatosis, Wilson's disease, primary biliary cirrhosis, alcoholic liver disease, autoimmune liver disease, inherited liver disease including alpha-1 antitrypsin deficiency, and hepatic venous outflow block. (8) Patients who consented to the study.

Table 1. Profiles and laboratory data at the initial visit of 1,181 patients infected with HCV, who had not received antiviral therapy

Demographic data	
Number of patients	1,181
Sex (male/female)	608/573
Age (years)*	60 (20-93)
History of blood transfusion	526 (49.2%)
Family history of liver disease	201 (20.3%)
Lifetime cumulative alcohol intake (>500 kg)	110 (10.8%)
Laboratory data*	
Total bilirubin (mg/dl)	0.7(0.1-20.0)
Aspartate aminotransferase (IU/l)	71 (13-1,052)
Alanine aminotransferase (IU/l)	88 (4-1,210)
Albumin (g/dl)	4.1 (1.0-5.5)
Hemoglobin (g/dl)	14.0 (7.8-18.0)
Platelet count ($\times 10^4/mm^3$)	15.3 (2.6-52.9)
HCV genotype (Ib / 2a or 2b)	750/431
Levels of viremia (high viral load)	757 (74.4%)
Amino acid substitutions in the HCV genotype Ib	
Core aa 70 (argjnine / glutamine (histidine))	431/319
Core aa 91 (leucine / methionine)	482/268

Data are number and percentages of patients, except those denoted by *, which represent the median (range) values.

Table 1 summarizes the profiles and laboratory data at the initial visit of 1,181 patients infected with HCV who had not received antiviral therapy. They did not receive antiviral therapy based on concerns about adverse effects, lack of time for treatment, physician recommendation based on the appearance of depression and cardiopulmonary disease, lower levels of aspartate aminotransferase (AST) / alanine aminotransferase (ALT), or elderly patients. They included 608 males and 573 females, aged 20 to 93 years (median, 60 years). The median follow-up time from the initial visit until death or until the last visit was 9.0 years (range, 0.0-37.7

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View this article online at wileyonlinelibrary.com.

DOI 10.1002/hep.25949

Potential conflict of interest: Norio Akuta has received speakers' bureau from MSD K.K., and holds a right to get some loyalty from SRL. Inc.. Hiromitsu Kumada has received speakers' bureau from MSD K.K., Mitsubishi Tanabe Pharma, Dainippon Sumitomo Pharma, Bristol-Myers Squibb, and holds a right to get some loyalty from SRL. Inc.. Fumitaka Suzuki has received speakers' bureau from Bristol-Myers Squibb. The other authors have nothing to disclose.

years). The study protocol was approved by the Human Ethics Review Committee of the institution.

Laboratory Investigations. Blood samples were frozen at −80°C within 4 hours of collection and were not thawed until used for testing. Anti-HCV, HCV RNA, HCV genotype, and aa substitutions of the HCV-1b core region were assayed using stored frozen sera. HCV genotype was determined by PCR using a mixed primer set derived from nucleotide sequences of the NS5 region.[24] HCV RNA quantitative analysis was measured by branched DNA assay v. 2.0 (Chiron), AMPLICOR GT HCV Monitor v. 2.0 using the 10-fold dilution method (Roche Molecular Systems, Pleasanton, CA), or COBAS TaqMan HCV test (Roche Diagnostics, Tokyo, Japan). High viral load of viremia levels was defined as branched DNA assay ≥1.0 Meq/mL, AMPLICOR GT HCV Monitor ≥100 × 10^3 IU/mL, or COBAS TaqMan HCV test ≥5.0 log IU/mL. Low viral load was defined as branched DNA assay <1.0 Meq/mL, AMPLICOR GT HCV Monitor <100 × 10^3 IU/mL, or COBAS TaqMan HCV test <5.0 log IU/mL.

Detection of Amino Acid Substitutions in Core Regions of HCV-1b. In the present study, aa substitutions of the core region of HCV-1b were analyzed by direct sequencing. HCV RNA was extracted from serum samples and reverse transcribed with random primer and MMLV reverse transcriptase (Takara Syuzo, Tokyo, Japan). Nucleic acids of the core region were amplified by nested PCR using the following primers. The first-round PCR was performed with CE1 (sense, 5′-GTC TGC GGA ACC GGT GAG TA-3′, nucleotides: 134-153) and CE2 (antisense, 5′-GAC GTG GCG TCG TAT TGT CG-3′, nucleotides: 1096-1115) primers, and the second-round PCR with CC9 (sense, 5′-ACT GCT AGC CGA GTA GTG TT-3′, nucleotides: 234-253) and CE6 (antisense, 5′-GGA GCA GTC GTT CGT GAC AT-3′, nucleotides: 934-953) primers. All samples were initially denatured at 95°C for 2 minutes. The 35 cycles of amplification were set as follows: denaturation for 30 seconds at 95°C, annealing of primers for 30 seconds at 55°C, and extension for 1 minute at 72°C with an additional 7 minutes for extension. Then 1 μL of the first PCR product was transferred to the second PCR reaction. Other conditions for the second PCR were the same as the first PCR, except that the second PCR primers were used instead of the first PCR primers. The amplified PCR products were purified by the QIA quick PCR purification kit (Qiagen, Tokyo, Japan) after agarose gel electrophoresis and then used for direct sequencing. Dideoxynucleotide termination sequencing was performed with the Big Dye Deoxy Terminator Cycle Sequencing kit (Perkin-Elmer, Tokyo, Japan).

With the use of HCV-J (accession no. D90208) as a reference,[25] the dominant sequence of 1-191 aa in the core protein of HCV-1b was determined by direct sequencing and then compared with the consensus sequence constructed on 50 clinical samples to detect substitutions at aa 70 of arginine (Arg70) or glutamine/histidine (Gln70/His70) and aa 91 of leucine (Leu91) or methionine (Met91).[6] Especially, patients were classified into three HCV subgroups according to HCV genotype in combination with aa substitutions in HCV-1b core region (HCV-1b of Arg70, HCV-1b of Gln70(His70), and HCV-2a/2b).

Determination of IL28B Genotype. IL28B rs8099917 was genotyped by the Invader assay, TaqMan assay, or direct sequencing, as described.[26,27]

Follow-Up and Diagnosis of HCC. Follow-up of patients was made on a monthly to trimonthly basis after the initial visit. Imaging diagnosis was made one or more times per year with ultrasonography, computed tomography, or magnetic resonance imaging. During this time, liver-related death, which included HCC, cholangiocellular carcinoma, liver failure, or esophageal variceal bleeding, was also evaluated.

Statistical Analysis. The cumulative rates of hepatocarcinogenesis, survival for liver-related death, and amino acid changes in the core region were calculated using the Kaplan-Meier technique; differences between the curves were tested using the log-rank test. Statistical analyses of hepatocarcinogenesis, survival, and amino acid changes, according to groups, were calculated using the period from the initial visit. Stepwise Cox regression analysis was used to determine independent predictive factors that were associated with hepatocarcinogenesis and survival for liver-related death. The hazard ratio (HR) and 95% confidence interval (95% CI) was also calculated. Potential predictive factors associated with hepatocarcinogenesis and survival for liver-related death included the variables: sex, age, history of blood transfusion, family history of liver disease, lifetime cumulative alcohol intake, total bilirubin, AST, ALT, albumin, hemoglobin, platelet count, levels of viremia, and HCV subgroup according to HCV genotype in combination with aa substitution in core region. Variables that achieved statistical significance ($P < 0.05$) on univariate analysis were tested by multivariate Cox proportional hazard model to identify significant independent factors. Statistical comparisons were performed using the SPSS software (Chicago, IL). $P < 0.05$ by the two-tailed test were considered significant.

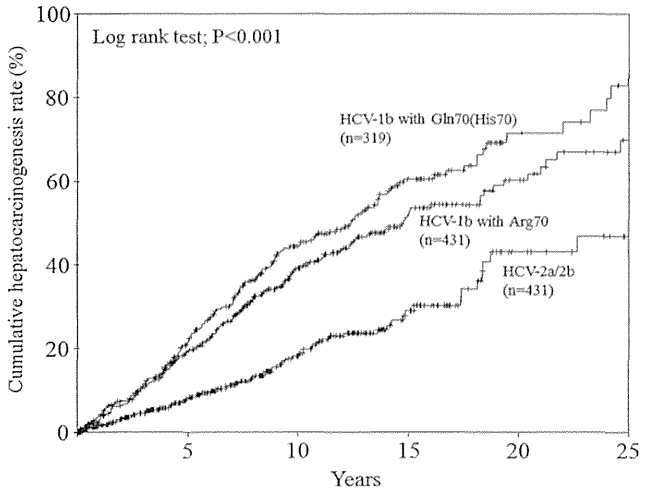

Fig. 1. Cumulative hepatocarcinogenesis rates according to HCV genotype in combination with amino acid substitutions in core region of HCV-1b. The rates were significantly different among the three HCV subgroups ($P < 0.001$; log-rank test). Especially, the rates in HCV-1b of Gln70(His70) were significantly higher than those in HCV-1b of Arg70 ($P = 0.028$; log-rank test) and HCV-2a/2b ($P < 0.001$; log-rank test), and the rates in HCV-1b of Arg70 were also significantly higher than those in HCV-2a/2b ($P < 0.001$; log-rank test).

Results

Hepatocarcinogenesis Rates and Survival Rates for Liver-Related Death in Patients Infected With HCV Who Had Not Received Antiviral Therapy. During the follow-up, 413 patients (35.0%) developed HCC. The cumulative hepatocarcinogenesis rates were 16.3, 34.3, 48.3, 58.7, and 69.1% at the end of 5, 10, 15, 20, and 25 years, respectively. The median interval between the initial visit and detection of HCC was 6.2 years (range, 0.1-31.7 years).

During the follow-up period, 243 patients (20.6%) died due to liver-related causes, and 97 of 243 (90.5%) developed HCC. The cumulative survival rates for liver-related death were 96.2, 84.8, 68.9, 55.0, and 46.1% at the end of 5, 10, 15, 20, and 25 years, respectively. The median interval between the initial visit and liver-related death was 10.1 years (range, 0.4-35.8 years).

Hepatocarcinogenesis Rates and Survival Rates for Liver-Related Death According to HCV Genotype in Combination with Amino Acid Substitutions in Core Region of HCV-1b. During the follow-up, 163 patients (51.3%), 175 (41.2%), and 75 (17.6%) developed HCC in HCV-1b of Gln70(His70), HCV-1b of Arg70, and HCV-2a/2b, respectively. In HCV-1b of Gln70(His70), HCV-1b of Arg70, and HCV-2a/2b, the cumulative hepatocarcinogenesis rates were 21.7, 19.3, 8.0% at the end of 5 years; 44.4, 39.4, 18.2% at the end of 10 years; 60.4, 52.7, 29.1% at the end of 15 years; 71.6, 60.3, 43.1% at the end of 20 years; and 87.1, 69.8, 46.9% at the end of 25 years, respectively. The rates were significantly different among the three HCV subgroups ($P < 0.001$) (Fig. 1). Especially, the rates in HCV-1b of Gln70(His70) were significantly higher than those in HCV-1b of Arg70 ($P = 0.028$) and HCV-2a/2b ($P < 0.001$), and the rates in HCV-1b of Arg70 were also significantly higher than those in HCV-2a/2b ($P < 0.001$).

During the follow-up, 104 patients (34.4%), 97 (23.4%), and 42 (10.0%) died due to liver-related causes in HCV-1b of Gln70(His70), HCV-1b of Arg70, and HCV-2a/2b, respectively. In HCV-1b of Gln70(His70), HCV-1b of Arg70, and HCV-2a/2b, the cumulative survival rates for liver-related death were 95.2, 95.4, 97.9% at the end of 5 years; 77.7, 83.3, 93.9% at the end of 10 years; 58.4, 68.4, 81.2% at the end of 15 years; 39.3, 58.4, 69.0% at the end of 20 years; and 33.8, 47.5, 59.5% at the end of 25 years, respectively. The rates were significantly different among the three HCV subgroups ($P < 0.001$) (Fig. 2). Especially, the rates in HCV-1b of Gln70(His70) were significantly lower than those in HCV-1b of Arg70 ($P = 0.016$) and HCV-2a/2b ($P < 0.001$), and the rates in HCV-1b of Arg70 were also significantly lower than those in HCV-2a/2b ($P < 0.001$).

Predictive Factors Associated with Hepatocarcinogenesis and Survival for Liver-Related Death in Patients Infected with HCV Who Had Not Received Antiviral Therapy. The data for the whole population

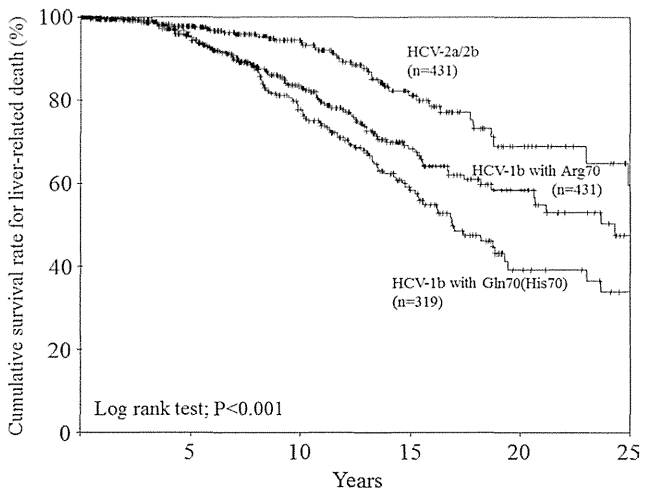

Fig. 2. Cumulative survival rates for liver-related death according to HCV genotype in combination with amino acid substitutions in the core region of HCV-1b. The rates were significantly different among the three HCV subgroups ($P < 0.001$; log-rank test). Especially, the rates in HCV-1b of Gln70(His70) were significantly lower than those in HCV-1b of Arg70 ($P = 0.016$; log-rank test) and HCV-2a/2b ($P < 0.001$; log-rank test), and the rates in HCV-1b of Arg70 were also significantly lower than those in HCV-2a/2b ($P < 0.001$; log-rank test).

Table 2. Factors associated with hepatocarcinogenesis in patients infected with HCV, who had not received antiviral therapy, identified bymultivariate analysis

[Factors]	[Category]	Hazard ratio (95% confidence interval)	P
Gender	1: female	1	
	2: male	1.78 (1.44-2.21)	<0.001
Age (years)	I:<60	1	
	2:≥60	1.68 (1.35-2.09)	<0.001
Albumin (g/dl)	1: ≥3.9	1	
	2: <3.9	1.94 (1.55-2.42)	<0.001
Platelet count ($\times 10^4/mm^3$)	1: ≥15.0	1	
	2:<15.0	2.89 (2.25-3.72)	<0.001
Aspartate aminotransferase (IU/l)	1:<67	1	
	2:≥67	1.92 (1.47-2.52)	<0.001
HCV subgroup	1: HCV-2a/2b	1	
	2: HCV-1b with Arg70	1.91 (1.42-2.55)	<0.001
	3: HCV-1b with Gln70(His70)	1.94 (1.45-2.61)	<0.001

Cox proportional hazard model

sample were analyzed to determine those factors that could predict hepatocarcinogenesis and survival for liver-related death.

Univariate analysis identified eight parameters that significantly correlated with hepatocarcinogenesis. These included gender (male; $P < 0.001$), age (≥60 years; $P < 0.001$), total bilirubin (≥1.2 mg/dL; $P < 0.001$), AST (≥67 IU/L; $P < 0.001$), ALT (≥85 IU/L; $P < 0.001$), platelet count (<15.0 × $10^4/mm^3$; $P < 0.001$), albumin (<3.9 g/dL; $P < 0.001$), and lifetime cumulative alcohol intake (≥500 kg; $P = 0.025$). Furthermore, the rates in HCV-1b of Gln70(His70) were significantly higher than those in HCV-1b of Arg70 ($P = 0.028$) and HCV-2a/2b ($P < 0.001$). These factors were entered into multivariate analysis, which then identified six parameters that significantly influenced hepatocarcinogenesis independently: gender (male; HR 1.78, $P < 0.001$), age (≥60 years; HR 1.68, $P < 0.001$), albumin (<3.9 g/dL; HR 1.94, $P < 0.001$), platelet count (<15.0 × $10^4/mm^3$; HR 2.89, $P < 0.001$), AST (≥67 IU/L; HR 1.92, $P < 0.001$), and HCV subgroup (HCV-1b of Gln70(His70); HR 1.94, $P = 0.001$) (Table 2).

Univariate analysis identified seven parameters that significantly correlated with survival for liver-related death. These included gender (male; $P < 0.001$), age (≥60 years; $P < 0.001$), total bilirubin (≥1.2 mg/dL; $P < 0.001$), AST (≥67 IU/L; $P < 0.001$), ALT (≥85 IU/L; $P < 0.001$), platelet count (<15.0 × $10^4/mm^3$; $P < 0.001$), and albumin (<3.9 g/dL; $P < 0.001$). Furthermore, the rates in HCV-1b of Gln70(His70) were significantly lower than those in HCV-1b of Arg70 ($P = 0.016$) and HCV-2a/2b ($P < 0.001$). These factors were entered into multivariate analysis, which then identified six parameters that significantly influenced survival for liver-related death independently: gender (male; HR 1.91, $P < 0.001$), age (≥60 years; HR 1.61, $P = 0.001$), albumin (<3.9 g/dL; HR 2.49, $P < 0.001$), platelet count (<15.0 × $10^4/mm^3$; HR 3.69, $P < 0.001$), AST (≥67 IU/L; HR 4.16, $P < 0.001$), and HCV subgroup (HCV-1b of Gln70(His70); HR 2.16, $P < 0.001$) (Table 3).

IL28B Genotype and Time-Dependent Amino Acid Changes in Core Region of HCV-1b. Among 1,181 patients, 359 could be evaluated for changes over time of dominant amino acid by direct sequencing in core aa 70 of HCV-1b. Furthermore, among 359 patients, 142 could also be analyzed for the relationship between *IL28B* rs8099917 genotype and time-dependent changes of core aa 70.

In 199 patients of Arg70 at the initial visit, 34 patients (17.1%) changed from Arg70 to Gln70(His70) during the follow-up. Inversely, in 160 patients of Gln70(His70) at the initial visit, eight patients (5.0%) changed from Gln70(His70) to Arg70 during the follow-up. In change from Arg70 to Gln70(His70), and change from Gln70(His70) to Arg70, the cumulative change rates were 3.0, 0% at the end of 5 years; 16.8, 5.8% at the end of 10 years; 27.4, 11.5% at the end of 15 years; and 38.9, 16.7% at the end of 20 years, respectively. The cumulative change rates from Arg70 to Gln70(His70) were significantly higher than those from Gln70(His70) to Arg70 ($P = 0.002$).

In 78 patients of Arg70 and TT genotype at the initial visit, nine (11.5%) changed from Arg70 to Gln70(His70) during the follow-up. In 11 patients of Arg70 and non-TT genotype at the initial visit, seven (63.6%) changed from Arg70 to Gln70(His70) during the follow-up. In TT and non-TT genotype, the cumulative change rates were 0, 9.1% at the end of 5 years; 3.2, 65.4% at the end of 10 years; 14.8, 65.4% at the end of 15 years; and 29.0, 65.4% at the end of 20 years, respectively. The cumulative change rates in non-TT genotype were significantly higher than those in TT genotype ($P < 0.001$) (Fig. 3A).

In 30 patients of Gln70(His70) and TT genotype at the initial visit, three patients (10.0%) changed from Gln70(His70) to Arg70 during the follow-up. In 23 patients of Gln70(His70) and non-TT genotype at the initial visit, no patients changed from Gln70(His70) to Arg70 during the follow-up. In TT and non-TT genotype, the cumulative change rates were 0, 0% at

Table 3. Factors associated with survival for liver-related death in patients infected with HCV, who had not received antiviral therapy, identified by multivariate analysis

[Factors]	[Category]	Hazard ratio (95% confidence interval)	P
Gender	1: female	1	
	2: male	1.91 (1.45-2.52)	<0.001
Age (years)	1:<60	1	
	2:≥60	1.61 (1.21-2.12)	0.001
Albumin (g/dl)	1:≥3,9	1	
	2:<3.9	2.49 (1.87-3.31)	<0.001
Platelet count ($\times 10^4/mm^3$)	1:≥15.0	1	
	2:<15.0	3.69 (2.65-5.13)	<0.001
Aspartate aminotransferase (IU/l)	1:<67	1	
	2:≥67	4.16 (2.43-7.11)	<0.001
HCV subgroup	1: HCV-2a/2b	1	
	2: HCV-1b with Arg70	1.83 (1.25-2.68)	0.002
	3: HCV-1b with Gln70(His70)	2.16 (1.48-3.16)	<0.001

Cox proportional hazard model

the end of 5 years; 9.1, 0% at the end of 10 years; 20.5, 0% at the end of 15 years; and 20.5, 0% at the end of 20 years, respectively. The cumulative change rates in TT genotype were not significantly higher than those in non-TT genotype ($P = 0.114$) (Fig. 3B).

Discussion

This is the first report to indicate that aa substitution in the core region might affect hepatocarcinogenesis and survival for liver-related death in HCV patients who had not received antiviral therapy. The treatment-resistant mechanism and oncogenic potential of HCV core region are still unclear. Moriishi et al.[28,29] showed that a knockout of the PA28γ gene induces the accumulation of HCV core protein in the nucleus of hepatocytes of HCV core gene transgenic mice and disrupts development of both hepatic steatosis and HCC. Hu et al.[13] indicated that the point-mutations of the core gene, including core aa 70 and aa 91, might change the secondary structure of not only RNA but also protein. As a result, the functions of both RNA and protein of the core region, such as an interaction with other DNA/RNA or proteins, might change and lead to hepatocarcinogenesis. Funaoka et al.[30] recently reported that treatment-resistant substitutions of core aa 70 and aa 91 (Gln70/His70 and Met91) were resistant to interferon *in vitro*, and the resistance might be induced by interleukin 6-induced upregulation of SOCS3. Further studies should be performed to investigate the treatment-resistant mechanism and oncogenic potential of aa substitution in the core region.

The association between HCV genotype and the risk of HCC is not clear. A previous report indicated that hepatocarcinogenesis rates in patients infected with HCV-1b were significantly higher than those in patients infected with HCV-2a/2c, based on an Italian cohort,[31] and this finding might be partly explained by distribution of HCV-1b of Arg70 or Gln70(His70). In fact, the hepatocarcinogenesis rates in HCV-1b of Gln70(His70) were significantly higher than those in HCV-1b of Arg70 and HCV-2a/2b in the present study based on a Japanese cohort. The present study is the first report to indicate that substitution of aa 70 in

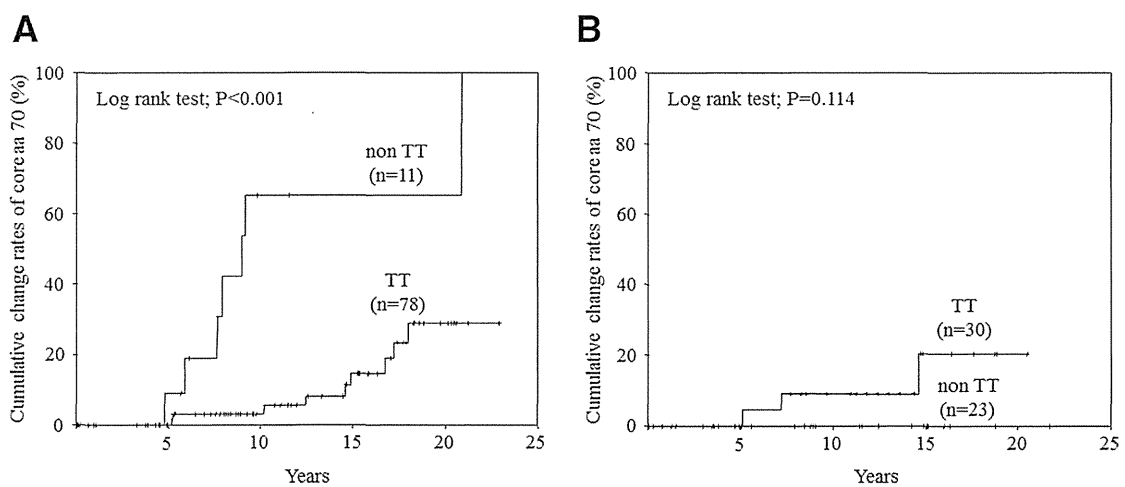

Fig. 3. Changes over time of dominant amino acid by direct sequencing in core aa 70 of HCV-1b, according to *IL28B* rs8099917 genotype. (A) In HCV-1b patients of Arg70 at the initial visit, cumulative change rates from Arg70 to Gln70(His70) during follow-up. The rates in non-TT genotype were significantly higher than those in TT genotype ($P < 0.001$; log-rank test). (B) In HCV-1b patients of Gln70(His70) at the initial visit, cumulative change rates from Gln70(His70) to Arg70 during follow-up. The rates in TT genotype were not significantly higher than those in non-TT genotype ($P = 0.114$; log-rank test).

the core region of HCV-1b is not only an important predictor of hepatocarcinogenesis, but also of survival for liver-related death in HCV patients who had not received antiviral therapy. The reason for the higher rates of liver-related death in HCV-1b of Gln70(His70) might be due to the higher rates of HCC. In conclusion, reducing the risk of hepatocarcinogenesis by HCV RNA eradication and/or ALT normalization by antiviral therapy should be recommended, especially in HCV-1b of Gln70(His70) as a high-risk group for hepatocarcinogenesis.[32]

The significant linkage between substitution of aa 70 and *IL28B* genotype had been shown,[21-23] but the mechanism of complex interaction between the virus and host is not clear. In the present study, the cumulative change rates from Arg70 to Gln70(His70) were significantly higher than those from Gln70(His70) to Arg70. Especially, the rates from Arg70 to Gln70(His70) in *IL28B* rs8099917 non-TT genotype were significantly higher than those in TT genotype. Although the molecular mechanisms of their relationship remain unknown, it could be speculated that *IL28B* genotype has an influence on the time-dependent changes of core aa 70, and refractory factors for treatment might accumulate in HCV-1b patients with non-TT. Hence, elucidating the relationship between substitution of aa 70 and *IL28B* genotype is an important step in understanding the mechanism of HCV treatment-resistance and disease progression.

The impact of *IL28B* genotype on hepatocarcinogenesis is controversial.[18-21] In this study, the effect of *IL28B* rs8099917 genotype on HCC was assessed in 515 of 2,799 consecutive HCV-infected patients who had not received antiviral therapy. Interestingly, the cumulative hepatocarcinogenesis rates in TT of the treatment-sensitive genotype was not significantly lower than those in non-TT of the treatment-resistant genotype ($P = 0.930$; log-rank test) in a preliminary study based on a small numbers of patients (Fig. 4). This result suggests that core aa 70 as a predictor of hepatocarcinogenesis might not only be influenced by *IL28B* genotype, but also by other factors strongly related to hepatocarcinogenesis independent of *IL28B* genotype. As a whole, it is regrettable that its impact on hepatocarcinogenesis in HCV patients who had not received antiviral therapy could not be investigated in this study. Further comprehensive studies should be performed to disclose the molecular mechanisms for the complicated relationships among core aa 70, *IL28B* genotype, and hepatocarcinogenesis.

The limitations of the present study are that patients who had received treatment besides IFN-related therapy (such as ursodeoxycholic acid, branched chain amino acid, and phlebotomy) could not be excluded. Furthermore, the clinical impact of metabolic factors (such as diabetes, insulin resistance, hepatocyte steatosis, and obesity) on hepatocarcinogenesis could also not be investigated. Further studies should be performed to investigate the clinical impact of treatment besides IFN-related therapy and metabolic factors on hepatocarcinogenesis.[33-37]

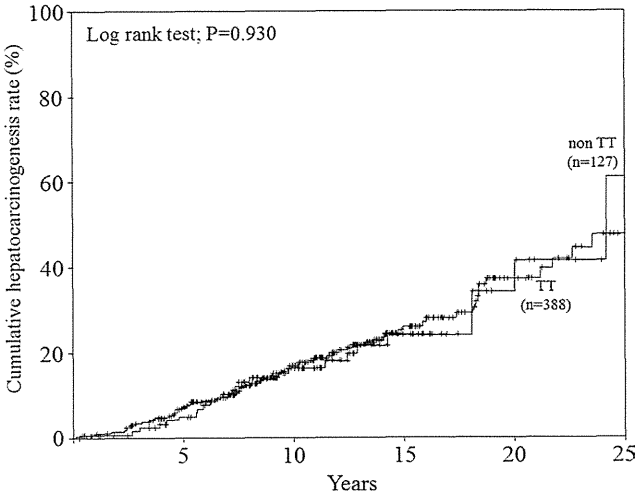

Fig. 4. Cumulative hepatocarcinogenesis rates according to *IL28B* rs8099917 genotype. The rates in TT genotype were not significantly lower than those in non-TT genotype ($P = 0.930$; log-rank test) in a preliminary study based on a small number of 515 patients.

In conclusion, substitution of aa 70 in the core region of HCV-1b is the important predictor of hepatocarcinogenesis and survival for liver-related death in HCV patients who had not received antiviral therapy. This study emphasizes the importance of antiviral therapy to reduce the risk of hepatocarcinogenesis, especially in HCV-1b of Gln70(His70) as a high-risk group for hepatocarcinogenesis. Furthermore, *IL28B* genotype might partly affect changes over time of dominant amino acid in core aa 70. This result should be interpreted with caution because races other than Japanese populations and patients infected with HCV-1a were not included. Any generalization of the results should await confirmation by studies of patients of other races and HCV-1a. Further prospective studies of a larger number of patients matched for race and HCV genotype are required to explore the relationship between core aa 70, *IL28B* genotype, and hepatocarcinogenesis.

References

1. Niederau C, Lange S, Heintges T, Erhardt A, Buschkamp M, Hürter D, et al. Progress of chronic hepatitis C: results of a large, prospective cohort study. HEPATOLOGY 1998;28:1687-1695.

2. Kenny-Walsh E. Clinical outcomes after hepatitis C infection from contaminated anti-D immune globulin. Irish Hepatology Research Group. N Engl J Med 1999;340:1228-1233.
3. Tsubota A, Arase Y, Someya T, Suzuki Y, Suzuki F, Saitoh S, et al. Early viral kinetics and treatment outcome in combination of high-dose interferon induction vs. pegylated interferon plus ribavirin for naive patients infected with hepatitis C virus of genotype 1b and high viral load. J Med Virol 2005;75:27-34.
4. Koike K. Molecular basis of hepatitis C virus-associated hepatocarcinogenesis: lessons from animal model studies. Clin Gastroenterol Hepatol 2005;3:S132-S135.
5. Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, et al. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat Med 1998;4:1065-1067.
6. Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, et al. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype1b high viral load and non-virological response to interferon-ribavirin combination therapy. Intervirology 2005;48:372-380.
7. Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. J Hepatol 2007;46:403-410.
8. Donlin MJ, Cannon NA, Yao E, Li J, Wahed A, Taylor MW, et al. Pretreatment sequence diversity differences in the full-length hepatitis C virus open reading frame correlate with early response to therapy. J Virol 2007;81:8211-8224.
9. Akuta N, Suzuki F, Hirakawa M, Kawamura Y, Yatsuji H, Sezaki H, et al. Amino acid substitution in hepatitis C virus core region and genetic variation near the interleukin 28B gene predict viral response to telaprevir with peginterferon and ribavirin. HEPATOLOGY 2010;52:421-429.
10. Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Amino acid substitutions in the hepatitis C virus core region are the important predictor of hepatocarcinogenesis. HEPATOLOGY 2007;46: 1357-1364.
11. Fishman SL, Factor SH, Balestrieri C, Fan X, Dibisceglie AM, Desai SM, et al. Mutations in the hepatitis C virus core gene are associated with advanced liver disease and hepatocellular carcinoma. Clin Cancer Res 2009;15:3205-3213.
12. Nakamoto S, Imazeki F, Fukai K, Fujiwara K, Arai M, Kanda T, et al. Association between mutations in the core region of hepatitis C virus genotype 1 and hepatocellular carcinoma development. J Hepatol 2010;52:72-78.
13. Hu Z, Muroyama R, Kowatari N, Chang J, Omata M, Kato N. Characteristic mutations in hepatitis C virus core gene related to the occurrence of hepatocellular carcinoma. Cancer Sci 2009;100:2465-2468.
14. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 2009;461:399-401.
15. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 2009;41:1105-1109.
16. Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, et al. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 2009;41:1100-1104.
17. Rauch A, Kutalik Z, Descombes P, Cai T, di Iulio J, Mueller T, et al., Swiss Hepatitis C and HIV Cohort Studies. Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure — a genome-wide association study. Gastroenterology 2010;138:1338-1345.
18. Fabris C, Falleti E, Cussigh A, Bitetto D, Fontanini E, Bignulin S, et al. IL-28B rs12979860 C/T allele distribution in patients with liver cirrhosis: role in the course of chronic viral hepatitis and the development of HCC. J Hepatol 2011;54:716-722.
19. Bochud P, Bibert S, Kutalik Z, Patin E, Guergnon J, Nalpas B, et al. IL28B alleles associated with poor HCV clearance protect against inflammation and fibrosis in patients infected with non-1 HCV genotypes. HEPATOLOGY 2012;55:384-394.
20. Joshita S, Umemura T, Katsuyama Y, Ichikawa Y, Kimura T, Morita S, et al. Association of IL28B gene polymorphism with development of hepatocellular carcinoma in Japanese patients with chronic hepatitis C virus infection. Hum Immunol 2012;73:298-300.
21. Miura M, Maekawa S, Kadokura M, Sueki R, Komase K, Shindo H, et al. Analysis of viral amino acids sequences and the IL28B SNP influencing the development of hepatocellular carcinoma in chronic hepatitis C. Hepatol Int 2011 Aug 17 [Epub ahead of print].
22. Abe H, Ochi H, Maekawa T, Hayes CN, Tsuge M, Miki D, et al. Common variation of IL28 affects gamma-GTP levels and inflammation of the liver in chronically infected hepatitis C virus patients. J Hepatol 2010;53:439-443.
23. Kobayashi M, Suzuki F, Akuta N, Sezaki H, Suzuki Y, Hosaka T, et al. Association of two polymorphisms of the IL28B gene with viral factors and treatment response in 1,518 patients infected with hepatitis C virus. J Gastroenterol 2012;47:596-605.
24. Chayama K, Tsubota A, Arase Y, Saitoh S, Koida I, Ikeda K, et al. Genotypic subtyping of hepatitis C virus. J Gastroenterol Hepatol 1993;8:150-156.
25. Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, et al. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc Natl Acad Sci U S A 1990;87:9524-9528.
26. 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;46:471-477.
27. Suzuki A, Yamada R, Chang X, Tokuhiro S, Sawada T, Suzuki M, et al. Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginie deminase 4, are associated with rheumatoid arthritis. Nat Genet 2003;34:395-402.
28. Moriishi K, Okabayashi T, Nakai K, Moriya K, Koike K, Murata S, et al. Proteasome activator PA28γ-dependent nuclear retention and degradation of hepatitis C virus core protein. J Virol 2003;77:10237-10249.
29. Moriishi K, Mochizuki R, Moriya K, Miyamoto H, Mori Y, Abe T, et al. Critical role of PA28γ in hepatitis C virus-associated steatogenesis and hepatocarcinogenesis. Proc Natl Acad Sci U S A 2007;104:1661-1666.
30. Funaoka Y, Sakamoto N, Suda G, Itsui Y, Nakagawa M, Kakinuma S, et al. Analysis of interferon signaling by infectious hepatitis C virus clones with substitutions of core amino acids 70 and 91. J Virol 2011; 85:5986-5994.
31. Bruno S, Crosignani A, Maisonneuve P, Rossi S, Silini E, Mondelli MU. Hepatitis C virus genotype 1b as a major risk factor associated with hepatocellular carcinoma in patients with cirrhosis: a seventeen-year prospective cohort study. HEPATOLOGY 2007;46:1350-1356.
32. Ikeda K, Saitoh S, Arase Y, Chayama K, Suzuki Y, Kobayashi M, et al. Effect of interferon therapy on hepatocellular carcinogenesis in patients with chronic hepatitis type C: a long-term observation study of 1643 patients using statistical bias correction with proportional hazard analysis. HEPATOLOGY 1999;29:1124-1130.
33. Tarao K, Fujiyama S, Ohkawa S, Miyakawa K, Tamai S, Hirokawa S, et al. Ursodiol use is possibly associated with lower incidence of hepatocellular carcinoma in hepatitis C virus-associated liver cirrhosis. Cancer Epidemiol Biomarkers Prev 2005;14:164-169.
34. Kawaguchi T, Sata M. Importance of hepatitis C virus-associated insulin resistance: therapeutic strategies for insulin sensitization. World J Gastroenterol 2010;16:1943-1952.
35. Kawaguchi T, Taniguchi E, Itou M, Sumie S, Yamagishi S, Sata M. The pathogenesis, complications and therapeutic strategy for hepatitis C virus-associated insulin resistance in the era of anti-viral treatment. Rev Recent Clin Trials 2010;5:147-157.
36. Kawaguchi T, Izumi N, Charlton MR, Sata M. Branched-chain amino acids as pharmacological nutrients in chronic liver disease. HEPATOLOGY 2011;54:1063-1070.
37. Sumida Y, Kanemasa K, Hara T, Inada Y, Sakai K, Imai S, et al. Impact of amino acid substitutions in the hepatitis C virus genotype 1b core region on liver steatosis and glucose tolerance in non-cirrhotic patients without overt diabetes. J Gastroenterol Hepatol 2011;26:836-842.

Exendin-4, a glucagon-like peptide-1 receptor agonist, modulates hepatic fatty acid composition and Δ-5-desaturase index in a murine model of non-alcoholic steatohepatitis

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Received December 20, 2013; Accepted May 28, 2014

DOI: 10.3892/ijmm.2014.1826

Abstract. Glucagon-like peptide-1 (GLP-1) is involved in the development of non-alcoholic steatohepatitis (NASH), which is characterized by fatty acid imbalance. The aim of this study was to investigate the effects of the GLP-1 receptor (GLP-1R) agonist, exendin-4 (Ex-4), on hepatic fatty acid metabolism and its key enzyme, Δ-5-desaturase, in a murine model of NASH. NASH was induced in db/db mice fed a methionine-choline deficient (MCD) diet. Ex-4 (n=4) or saline [control (CON); n=4] was administered intraperitoneally for 8 weeks. Steatohepatitis activity was evaluated by non-alcoholic fatty liver disease (NAFLD) activity score. Hepatic fatty acid composition and Δ-5-desaturase index were analyzed by gas chromatography. Ex-4 treatment significantly reduced body weight and the NAFLD activity score. Hepatic concentrations of long-chain saturated fatty acids (SFAs) were significantly higher in the Ex-4 group compared to the CON group (23240±955 vs. 31710±8436 μg/g•liver, P<0.05). Ex-4 significantly reduced hepatic n-3 polyunsaturated fatty acid (PUFA)/n-6 PUFA ratio compared to the CON group (13.83±3.15 vs. 8.73±1.95, P<0.05). In addition, the hepatic Δ-5-desaturase index was significantly reduced in the Ex-4 group compared to the CON group (31.1±12.4 vs. 10.5±3.1, P<0.05). In conclusion, the results showed that Ex-4 improved steatohepatitis in a murine model of NASH. Furthermore, Ex-4 altered hepatic long-chain saturated and PUFA composition and reduced the Δ-5-desaturase index. Thus, Ex-4 may improve NASH by regulating hepatic fatty acid metabolism.

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Abbreviations: GLP-1, glucagon-like peptide-1; NASH, non-alcoholic steatohepatitis; Ex-4, exendin-4; GLP-1R, GLP-1 receptor; NAFLD, non-alcoholic fatty liver disease; PUFA, polyunsaturated fatty acid; MCD, methionine-choline deficient; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid

Key words: incretin, exenatide, steatosis, fatty acid composition, fatty acid desaturase

Introduction

The incidence of non-alcoholic steatohepatitis (NASH) is rapidly increasing worldwide (1-4). NASH can be caused by various pathogenic mechanisms, including overeating, physical inactivity, diabetes mellitus, and medications (5,6). The gut directly links to the liver through the portal vein and is involved in the development of NASH (7,8). The gut secretes various hormones in the portal vein and regulates hepatic metabolism (9-11). Glucagon-like peptide-1 (GLP-1) is a gut hormone and is known to affect lipid metabolism in hepatocytes (9,11).

Exendin-4 (Ex-4) is a long-acting GLP-1 receptor (GLP-1R) agonist. GLP-1R occurs in the pancreatic islets, kidney, lung, heart, stomach, intestine, thyroid gland, and numerous regions of the peripheral and central nervous system (12-14). GLP-1R also occurs in hepatocytes, and treatment with Ex-4 substantially reduces triglyceride stores in hepatoma cells (15). Similarly, GLP-1R agonist reduces steatosis severity in certain animal models of NASH (16-19). Findings of previous studies have also shown that reduced hepatic accumulation of triglycerides is mediated by GLP-1R agonist upregulation of hepatic 3-phosphoinositide-dependent kinase-1 activity, protein kinase C ζ activity, peroxisome proliferator-activated receptor α activity, and fatty acid β-oxidation (15-19).

Fatty acids are an important triglyceride component. Fatty acids are a substrate of β-oxidation and yield large quantities of adenosine 5'-triphosphate (20). In addition, some polyunsaturated fatty acids (PUFAs) are a source of eicosanoids, which are biologically active substances. n-3 PUFAs are precursors of anti-inflammatory eicosanoids, including leukotriene B5, prostaglandin E3, and thromboxane B3 (21). On the other hand, n-6 PUFA are precursors of pro-inflammatory eicosanoids, including leukotriene B4, prostaglandin E2, and thromboxane B2 (21). A reduced n-3/n-6 PUFA ratio is a risk factor for chronic inflammatory diseases such as cardiovascular disease, inflammatory bowel disease, rheumatoid arthritis, and

NASH (22-24). Thus, besides quantitative abnormality in fatty acids, qualitative abnormality in fatty acids is an important pathogenesis of NASH.

The production of pro- and anti-inflammatory eicosanoids is regulated by desaturases, which are rate-limiting enzymes of n-3 and n-6 PUFA cascades (25). Δ-5-desaturase, also known as fatty acid desaturase 1, removes two hydrogen atoms from dihomo γ-linolenic acid and synthesizes arachidonic acid. Upregulation of Δ-5-desaturase activity promotes the production of pro-inflammatory eicosanoids (26). Notably, single-nucleotide polymorphisms in the Δ-5-desaturase gene are associated with circulating high sensitivity C-reactive protein levels in healthy young adults (27). Moreover, Δ-5-desaturase activity is associated with aging (28), development of type 2 diabetes mellitus (29), and NASH (30). However, the effects of Ex-4 on hepatic fatty acid composition and Δ-5-desaturase activity remain unclear.

The aim of this study was to investigate the effects of Ex-4 on severity of steatohepatitis, hepatic fatty acid composition, and Δ-5-desaturase index in a murine model of NASH.

Materials and methods

Materials. Reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) unless otherwise indicated.

Animals. NASH was induced in db/db mice fed a methionine-choline deficient (MCD) diet (31). Briefly, 5-week-old male db/db mice (BKS.Cg- + Leprdb/+Leprdb/Jcl®) weighing 15-20 g were purchased from CLEA Japan, Inc. (Tokyo, Japan). The mice were housed individually in an air-conditioned room at 22±3˚C and 55±10% humidity with a 12-h light/dark cycle. The mice were fed a normal diet during a 1-week quarantine and acclimatization period, followed by the MCD diet (CLEA Japan, Inc.) and water *ad libitum* throughout the experimental period. All the rat experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the University of Kurume Institutional Animal Care and Use Committee.

Treatment. Ex-4 (20 μg/kg; no. 24463, AnaSpec, Inc., Fremont, CA, USA) (Ex-4 group; n=4) or saline [control (CON) group; n=4] was administered intraperitoneally under anesthesia every morning for 8 weeks. At week 14, the mice were sacrificed by using ether anesthesia and the livers were obtained under anesthesia.

Measurement of body weight. Body weight was measured weekly, in the morning, through week 14.

Liver histology. Random histological sampling was performed throughout this study as previously described (32,33). Liver samples were fixed overnight in 10% buffered formalin and embedded in paraffin. All sections were cut at a thickness of 5 μm and stained with hematoxylin and eosin (H&E) (34,35).

Hepatic triglyceride content. Liver samples were fixed overnight in 10% buffered formalin. Sections were transferred to 70% ethanol and stained with Sudan IV (0.1% Sudan IV dissolved in equal parts acetone and 70% ethanol) to evaluate triglyceride content (36).

Non-alcoholic fatty liver disease (NAFLD) activity. NAFLD activity was evaluated by the NAFLD activity score, in which the following findings were evaluated semi-quantitatively: steatosis (0-3 points), lobular inflammation (0-2 points), hepatocellular ballooning (0-2 points), and fibrosis (0-4 points) (37).

Fatty acid composition. Total liver fatty acids were extracted according to Folch *et al* (38). Fatty acid methyl esters were isolated and quantified by gas chromatography furnished with a flame-ionization detector. The fatty acids measured (and expressed as μg/g•liver) were: lauric, myristic, myristoleic, palmitic, palmitoleic, stearic, oleic, linoleic, γ-linolenic, linolenic, arachidic, eicosenoic, eicosadienoic, 5,8,11-eicosatrienoic, dihomo γ-linolenic, arachidonic, eicosapentaenoic, behenic, erucic, docosatetraenoic, docosapentaenoic, lignoceric, docosahexaenoic, and nervonic acid.

Classification of fatty acids. Fatty acids were classified as follows: saturated fatty acids (SFAs), the sum of all identified SAFs; atherogenic SFAs, the sum of lauric, myristic, and palmitic acids; thrombogenic SFAs, the sum of myristic, palmitic, and stearic acids; medium SFAs, the sum of SFAs containing 11-16 carbon atoms; long SFAs, the sum of SFAs containing ≥16 carbon atoms; monounsaturated fatty acids (MUFAs), the sum of all identified MUFAs; PUFAs, the sum of all identified PUFAs; n-3 PUFAs, the sum of n-3 series PUFAs; n-6 PUFAs, the sum of n-6 series PUFA; Δ-5-desatulase index, arachidonic acid/γ-linolenic acid.

Statistical analysis. Data were expressed as mean ± SD. Differences between two groups were analyzed by the Wilcoxon test (JMP version 10.0.2, SAS Institute, Inc., Cary, NC). $P \leq 0.05$ was considered statistically significant.

Results

Effects of Ex-4 on body weight, appearance, and macroscopic appearance of the liver. In the CON group, body weight gradually increased to ~50 g at week 14 (Fig. 1A). In the Ex-4 group, body weight gain stopped 1 week after the Ex-4 treatment and reached a plateau at ~40 g at week 7 (Fig. 1A). Ex-4 significantly suppressed weight gain in MCD-fed db/db mice.

Representative mice from the CON and Ex-4 groups are shown in Fig. 1B. The mouse from the Ex-4 group was smaller and had a good coat of fur in comparison to the mouse from the CON group (Fig. 1B).

A representative macroscopic image of the liver of CON and Ex-4 mice is shown in Fig. 1C. CON livers exhibited xanthochromia with swelling, while the Ex-4 livers were brown, with no swelling (Fig. 1C).

Effects of Ex-4 on hepatic histology, hepatic triglyceride content, and the NAFLD activity score. Representative images of hepatic histology and Sudan IV staining are shown in Fig. 2A. Steatosis, lobular inflammation, and hepatocyte ballooning were milder in the Ex-4 group compared to the CON group (Fig. 2A). Obvious hepatic fibrosis was not evident

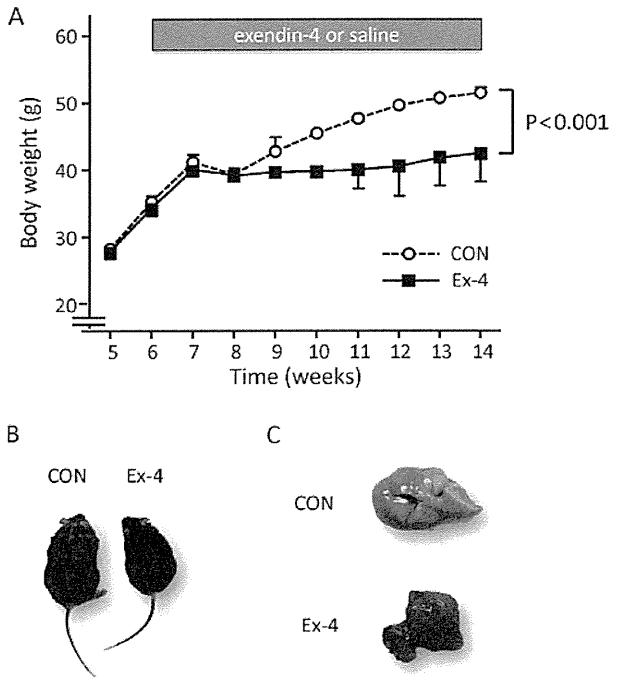

Figure 1. Effects of exendin-4 (Ex-4) on body weight, appearance, and macroscopic appearance of liver. (A) Changes in body weight. (B) Representative mice in the control (CON) and Ex-4 groups. (C) Representative livers from the CON and Ex-4 groups. P≤0.05 was considered statistically significant.

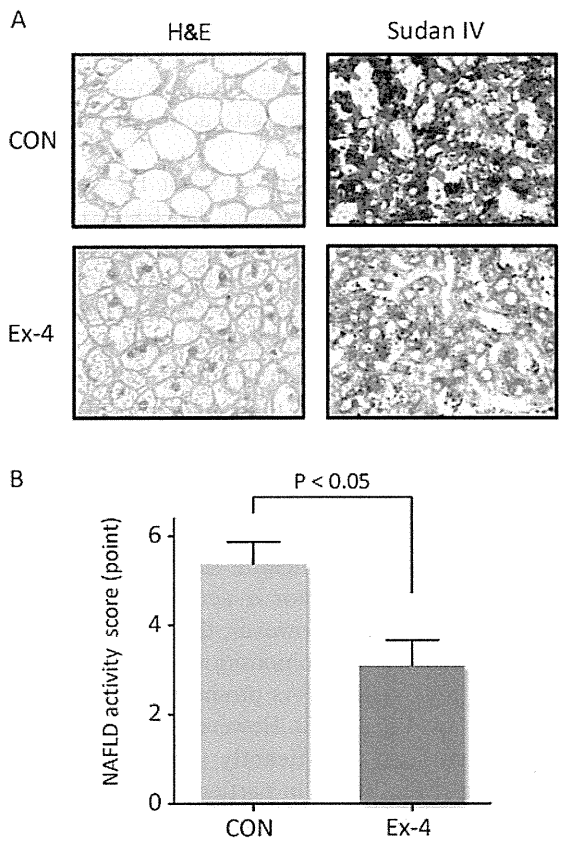

Figure 2. Effects of exendin-4 (Ex-4) on hepatic histology, hepatic triglyceride content, and non-alcoholic fatty liver disease (NAFLD) activity score. (A) Representative images of hepatic histology and Sudan IV staining. (B) The difference in the NAFLD activity score between the Ex-4 and control (CON) groups. P≤0.05 was considered statistically significant. Hematoxylin and eosin staining (H&E).

Table I. Effects of Ex-4 on hepatic SFA.

SFA type	Unit	CON	Ex-4	P
SFA	μg/g•liver	17838±3248	27541±9273	N.S.
Atherogenic	μg/g•liver	13414±2981	22457±8670	N.S.
Thrombogenic	μg/g•liver	17605±3244	27210±9260	N.S.
Medium-chain	μg/g•liver	15233±3554	25186±9799	N.S.
Long-chain	μg/g•liver	23240±955	31710±8436	<0.05

Ex-4, exendin-4; SFA, saturated fatty acid; CON, control; N.S., not significant.

Table II. Effects of Ex-4 on hepatic MUFAs and PUFAs.

Acid type	Unit	CON	Ex-4	P
MUFA	μg/g•liver	20355±6701	34965±14485	N.S.
PUFA	μg/g•liver	18410±791	25986±8050	<0.05
n-3 PUFA	μg/g•liver	2218.5±415.8	1992.4±288.7	N.S.
n-6 PUFA	μg/g•liver	16166±943	23937±7845	<0.05
n-3 PUFA/ n-6 PUFA	Ratio	13.83±3.15	8.73±1.95	<0.05

Ex-4, exendin-4; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; CON, control; N.S., not significant.

in either group. Hepatic triglyceride content was depleted in the Ex-4 group in comparison to the CON group (Fig. 2A).

The NAFLD activity score was significantly lower in the Ex-4 group than in the CON group (Fig. 2B).

Effects of Ex-4 on hepatic SFA. There was no significant difference in the hepatic SFA content of the CON and Ex-4 groups (Table I). No significant difference between the groups was observed in the hepatic content of atherogenic, thrombogenic, and medium-chain SFA. However, long-chain SFA content was significantly higher in the Ex-4 group compared to the CON group (Table I).

We also examined the hepatic content of each long-chain SFA component and found no significant differences in the palmitic, stearic, behenic, and lignoceric acid. However, hepatic arachidic acid was significantly higher in the Ex-4 group compared to the CON group (Fig. 3A-E).

Effects of Ex-4 on hepatic MUFAs and PUFAs. Hepatic MUFA content did not significantly differ between groups (Table II). However, hepatic PUFA content was significantly higher in the Ex-4 group compared to the CON group. Similarly, hepatic n-6 PUFA content and the n-3 PUFA/n-6 PUFA ratio were significantly higher in the Ex-4 group compared to the CON group (Table II).

We also assessed the hepatic content of each n-6 PUFA component and found no significant difference in arachidonic acid. However, the hepatic content of linoleic acid, γ-linolenic acid, and dihomo γ-linolenic acid was significantly higher in the Ex-4 group compared to the CON group (Fig. 4A-D). By contrast, hepatic Δ-5-desaturase index in the Ex-4 group