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Original Article

Difference in malignancies of chronic liver disease due to non-alcoholic fatty liver disease or hepatitis C in Japanese elderly patients

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Aim: Malignancies that include hepatocellular carcinoma often occurred in patients with chronic liver disease. The aim of this retrospective match control study was to assess the cumulative development incidence and predictive factors for total malignancies in elderly Japanese patients with non-alcoholic hepatic diseases (NAFLD) or hepatitis C virus (HCV).

Methods: A total of 1600 NAFLD patients with age of ≥60 years were enrolled, and 1600 HCV patients with age of ≥60 years were selected as control by matching 1:1 with NAFLD group for age, sex, and follow-up period. The primary goal is the first development of malignancies. Evaluation was performed by the use of the Wilcoxon rank sum test, the Kaplan–Meier method, and Cox proportional hazard model. The mean observation period is 8.2 years in both NAFLD and HCV group, respectively.

Results: The number of patients with the development of malignancies was 167 in the NAFLD group and 395 in the HCV group. The 10th development rate of malignancies was 13.9% in the NAFLD group and 28.2% in the HCV group (risk ratio 2.27; $P < 0.001$). The incident rates of hepatocellular carcinoma in all the malignancies were 6.0% (10/167) in the NAFLD group and 67.6% (267/395) in the HCV group ($P < 0.001$). The malignancies in the NAFLD group were observed in the following order: gastric cancer 34 cases (20.4%) > colon cancer 31 cases (18.6%) > prostate cancer 21 cases (12.6%).

Conclusions: The incident rates of hepatocellular carcinoma in all the malignancies were approximately 6% in the NAFLD group and two-thirds in the HCV group.

Key words: carcinogenesis, hepatitis C virus, non-alcoholic fatty liver disease

INTRODUCTION

NON-ALCOHOLIC FATTY LIVER disease (NAFLD) is one of the more common causes of chronic liver disease worldwide.[1–6] NAFLD is considered to be the liver component of metabolic syndrome.[7,8] It is associated with obesity, dyslipidemia, pituitary dysfunction, hypertension, sleep apnea, and diabetes mellitus type 2

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(T2DM).[9–13] In addition, NAFLD sometimes progressed to non-alcoholic steatohepatitis (NASH). In patients with cirrhotic NASH, liver-related events such as hepatocellular carcinoma (HCC) and liver failure are one of the main causes of morbidity and mortality.[14] However, studies on prolonged prognosis of NAFLD are few in Japan. Thus, the true prevalence and natural history of NAFLD in Japanese patients are still unclear.

On the other hand, hepatitis C virus (HCV) often causes liver cirrhosis and HCC.[15–18] The majority of HCC is ascribed to hepatitis viruses, of which 70–80% corresponding to approximately 35 000 per year is attributed to the persistent infection with HCV in Japan. However, studies on malignancies other than HCC are few in the HCV patients.

With this background in mind, the present study was initiated to investigate the cumulative incidence and risk factors of malignancies that includes HCC after prolonged follow-up in elderly Japanese patients with NAFLD or HCV. The strengths of the current study are the large numbers of patients included and the long-term follow-up of patients.

METHODS

Patients

THE NUMBER OF patients who were diagnosed with fatty liver by the ultrasonography (US) between January 1994 and December 2007 in the Health Management Center and/or Department of Hepatology, Toranomon Hospital, Tokyo, Japan was 10 810. Of these, 1600 Japanese patients satisfied the following enrolled criteria; (i) age of ≥60 years; (ii) daily alcohol intake of <20 g/day; (iii) negativity for hepatitis B surface antigens (HBsAg), hepatitis C virus antibodies, antinuclear antibodies, or antimitochondrial antibodies in serum, as determined by radioimmunoassay, enzyme-linked immunosorbent assay or indirect immunofluorescence assay; (iv) the absence of malignancies by gastrofiberscope, abdominal US, chest X-ray, and/or chest computed tomography (CT); (v) annual examination for health screening; and (vi) no underlying systemic disease, such as systemic lupus erythmatosus, rheumatic arthritis. Patients with either of the following criteria were excluded from the study: (i) they had illnesses that could seriously reduce their life expectancy; and (ii) they had history of carcinogenesis. In the same period, 7189 HCV patients without fatty liver determined by US were followed in the same hospital. Seven inclusion criteria and two exclusion criteria described in NAFLD group were applied to 2575 of these 7189 HCV patients without fatty liver. Thus, a total of 1600 NAFLD patients with age of ≥60 years were enrolled, and 1600 HCV patients with age of ≥60 years were selected as controls by matching 1:1 with the NAFLD group for age, sex, and follow-up period.

Patients were classified into three groups according to fasting plasma glucose (FPG): (i) those with FPG level of <109 mg/dL (normal glucose group); (ii) those with FPG level of 109–125 mg/dL (pre-diabetes group); and (iii) those with FPG level of ≥126 mg/dL (diabetes group).[19] Patients were regarded as hypertensive by the confirmation of blood pressure ≥140 mmHg systolic and/or ≥90 mmHg diastolic on at least three visits. We considered persons smokers if they had smoked a cigarette at the initiation of follow-up.

The primary goal is the development of malignancies. The diagnosis of malignancies was made due to tumor marker, imaging (US, CT or magnetic resonance imaging [MRI]), and/or histological examination.[20–27] All of the studies were performed retrospectively by collecting and analyzing data from the patient records. This study had been approved by the Institutional Review Board of our hospital.

Medical evaluation

Diagnosis of fatty liver was based on the presence of an ultrasonographic pattern consistent with bright liver with stronger echoes in the hepatic parenchyma than in the renal parenchyma.[28] US test was performed with a high-resolution, real-time scanner (model SSD-2000; Aloka Co., Ltd, Tokyo Japan. Mode Logic-700 MR; GE-Yokokawa Medical Systems, Tokyo, Japan). 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 the body mass index (BMI) was calculated as weight (in kg)/height (in m^2). All the 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.

Laboratory investigation

Anti-HCV was detected using a second-generation enzyme-linked immunosorbent assay (ELISA II) (Abbott Laboratories, North Chicago, IL, USA). HCV-RNA was determined by the Amplicor method (Cobas Amplicor HCV Monitor Test, v2.0, Roche, Tokyo,

Japan). HBsAg was tested by radioimmunoassay (Abbott Laboratories, Detroit, MI, USA). The used serum samples were stored at −80°C at the first consultation. Diagnosis of HCV infection was based on detection of serum HCV antibody and positive RNA.

Follow-up

We used 60 years of age as the starting point for observations in 1417 patients (NAFLD, 694 patients; HCV, 723 patients) who came to our hospital before the age of 60. In 1783 patients (NAFLD, 906 patients; HCV, 877 patients) who came after the age of 60, the day of first visit was used as the start of observations. All patients were followed up at least twice a year by monitoring hematological and biochemical data. Imaging examinations were done approximately once a year for each patient, using abdominal-US and Chest X-ray. Moreover, the patients were checked for tumor marker (carcinoembryonic antigen [CEA], α-fetoprotein [AFP], and prostate-specific antigen [PSA]), gastrofiberscope (or gastrography), and occult blood test of feces at least one year. Two hundred and eighty-two patients were lost to follow-up. Because the appearance of malignancy was not identified in these 282 patients, they were considered as censored data in statistical analysis.[29] Patients treated with antiviral agents were regarded as withdrawals at the time of having the negativity of HCV RNA level by the Amplicor method.

Statistical analysis

Clinical differences between the NAFLD group and HCV group were evaluated by Wilcoxon rank sum test or Fisher's exact test. The cumulative development rates of malignancies were calculated by using the Kaplan–Meier technique, and differences in the curves were tested using the log-rank test.[30] Independent risk factors associated with malignancies were studied using the stepwise Cox regression analysis.[31] The following 15 variables were analyzed for potential covariates for incidence of primary goals in NAFLD group and HCV group: age, gender, body mass index, hypertension, current smoking, albumin, triglyceride, total cholesterol, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltransferase (GGT), fasting plasma glucose, platelet, and AFP at the initiation time of follow-up. A *P*-value of less than 0.05 was considered significant. Data analysis was performed using the computer program SPSS package (SPSS 11.5 for Windows, SPSS, Chicago, IL, USA).

RESULTS

Characteristics of the patients enrolled

TABLE 1 SHOWS the baseline characteristics of the 1600 patients in NAFLD group and the 1600 patients in the HCV group at the initiation of follow-up. There are significant differences in several baseline characteristics such as body mass index, AST, ALT, triglyceride, total cholesterol, fasting plasma glucose, platelet, AFP between the HCV group and NAFLD group as shown in Table 1.

Development of malignancy

A total of 562 subjects (167 in NAFLD group and 395 in HCV group) developed malignancy during follow-up. The cumulative development rate of carcinogenesis at the 10th year was determined to be 13.9% in the NAFLD group and 28.2% in the HCV group by the use of the Kaplan–Meier method (Fig. 1). The development rate of each malignancy in both groups is shown in Table 2. The malignancies in the NAFLD group were observed in the following order: gastric cancer 34 cases (20.4%) > colon cancer 31 cases (18.6%) > prostate cancer 21 cases (12.6%). On the other hand, HCC in the HCV group accounted for two-thirds of malignancy. The development rates per 1000 person years in HCC and malignant lymphoma in the HCV group was statistically higher than those in the NAFLD group. However, there were no significant differences in gastric cancer, colon cancer, prostate cancer, and lung cancer between both groups. The incidence rates of HCC in all of the malignancies were 6.0% (10/167) in the NAFLD group and 67.6% (267/395) in the HCV group ($P < 0.001$). Seven of 10 NAFLD patients with development of HCC were evaluated as having histological liver condition at the time of development of HCC. One patient had simple steatosis, and another six patients had non-alcoholic steatohepatitis (NASH). The grade of liver fibrosis in six NASH patients with development of HCC was as follows: grade 1, one patient; grade 2, two patients; grade 3, two patients; grade 4, one patient.

The development rates of each malignancy between the NAFLD group and the HCV group based on the difference of gender are shown in Table 3. The development rates of HCC expressed by 1000 person years in the HCV group were two orders of magnitude higher than those in the NAFLD group in both males and females. There were no significant differences in other malignancies except for HCC between the

Table 1 Patient characteristics at the starting time of follow up†

	NAFLD group	HCV group	*P*-value
n	1600	1600	
Age (years)	62.5 ± 9.5	62.6 ± 8.7	0.936
Gender (male/female)	1200/400	1200/400	1.000
Body mass index	25.1 ± 2.6	21.8 ± 4.0	<0.001
Blood pressure			
(systolic, mmHg)	132 ± 17	133 ± 18	0.972
(diastolic, mmHg)	76 ± 11	77 ± 12	0.937
Hypertension (+/−)	279/1321	306/1294	0.252
Smoking (+/−)	421/1179	396/1141	0.807
AST (IU/L)	29 ± 15	77 ± 64	<0.001
ALT (IU/L)	37 ± 25	104 ± 97	<0.001
GGT (IU/L)	73 ± 79	83 ± 97	0.196
Albumin (g/dL)	4.2 ± 0.3	4.1 ± 0.4	0.883
Triglyceride (mg/dL)	161 ± 105	99 ± 51	<0.001
Total cholesterol (mg/dL)	211 ± 33	176 ± 38	<0.001
FPG (mg/dL)	104.1 ± 10.5	95.8 ± 9.3	<0.001
FPG (DM/pre-DM /normal)	208/330/1062	184/276/1140	<0.001
Platelet ($\times 10^4/mm^3$)	22.1 ± 6.5	15.8 ± 5.8	<0.001
AFP (ng/mL)	3.4 ± 2.4	10.8 ± 10.0	<0.001
Follow-up period (year)	8.2 ± 3.8	8.2 ± 3.9	0.928

†Data are number of patients or mean ± standard deviation.
AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DM, diabetes mellitus, FPG, fasting plasma glucose; GGT, gamma-glutamyltransferase; HCV, hepatitis C virus; NAFLD, non-alcoholic fatty liver disease.

NAFLD group and the HCV group in both males and females.

Predictive factors for the development of malignancies

The factors associated with the development of malignancies in the NAFLD group and HCV group are shown in Tables 4 and 5. In the NAFLD group, multivariate Cox proportional hazards analysis shows that malignancies occurred when patients had an age of ≥70 years (hazard ratio [HR] : 2.10; 95%CI = 1.38–3.17; $P < 0.001$), current smoking (HR : 1.64; 95%CI = 1.18–2.27; $P = 0.003$), and elevated glucose level (HR : 1.32; 95%CI = 1.08–1.61; $P = 0.007$).

On the other hand, in HCV group, multivariate Cox proportional hazards analysis shows that malignancies development rate was high with statistical significance when patients had elevated AFP (HR : 2.52; 95%CI = 1.94–3.44; $P < 0.001$), elevated glucose level (HR : 1.35; 95%CI = 1.18–1.59; $P < 0.001$), elevated AST level (HR : 1.75; 95%CI = 1.13–2.70; $P = 0.010$), hypoalbuminemia (HR : 1.51; 95%CI = 1.15–1.97; $P = 0.002$), male (HR : 1.49; 95%CI = 1.16–1.94; $P = 0.002$), and thrombocytopenia (HR : 1.49; 95%CI = 1.14–1.96; $P = 0.002$).

DISCUSSION

THE DEVELOPMENT INCIDENCE of malignancies in elderly patients with NAFLD or HCV has been described in the present study. The reason for selecting elderly patients is that development of malignancies in patients with age of ≥60 years occur frequently compared with young patients. Thus, it is likely that the difference between NAFLD and HCV patients tends to become clear.

The present study shows several findings with regard to the development of malignancies in elderly Japanese patients with NAFLD or HCV. First, HCC in the NAFLD group accounted for approximately 6% of the cause of malignancies. The four malignancies of the stomach, colon, prostate, and lung accounted for about 60% in the NAFLD group. Matsuda *et al.* have reported the cancer incidence in Japan.[32] According to their report, the outbreak of malignancies in a Japanese male popu-

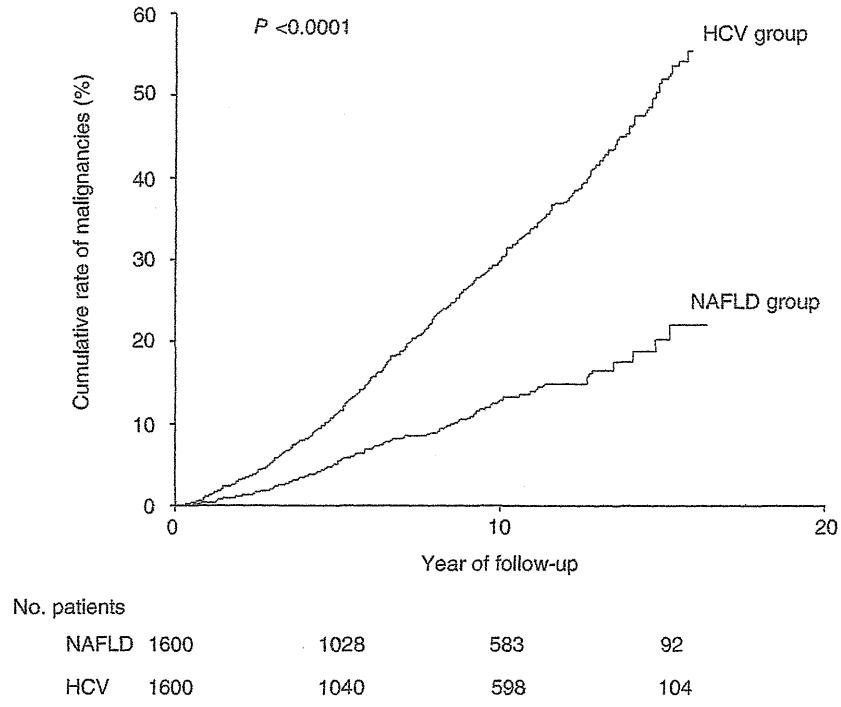

Figure 1 Cumulative development rate of malignancies in non-alcoholic hepatic diseases (NAFLD) or hepatitis C virus (HCV) patients.

lation was observed in the following order in 2005: gastric cancer 20.4% > colon cancer 16.0% > lung cancer 15.4% > prostatic cancer 10.9% > HCC 7.4%. On the other hand, the outbreak of malignancies in a Japanese female population was observed in the following order in 2005: mammary cancer 18.0% > colon cancer 16.2% > gastric cancer 13.6% > lung cancer 9.3% > uterine cancer 6.8%. The incidence of prostate cancer in NAFLD was greater than that in a total Japanese population. Renehan *et al.* showed that body mass index is connected with prostate carcinogenesis relative to other tumours.[33] NAFLD patients might tend to have

Table 2 Development rate of each malignancy in the non-alcoholic fatty liver disease (NAFLD) group and the hepatitis C virus (HCV) group†

Malignancies	NAFLD group		HCV group		*P*‡
	n (%)†	1000 person years	*n* (%)†	1000 person years	
Total	167 (100%)	12.96	395 (100%)	30.88	<0.001
Hepatocellular carcinoma	10 (6.0%)	0.78	267 (67.9%)	20.86	<0.001
Gastric cancer	34 (20.4%)	2.66	28 (7.1%)	2.19	0.522
Colon cancer	31 (18.6%)	2.42	26 (6.6%)	2.03	0.593
Prostate cancer	21 (12.6%)	1.64	14 (3.5%)	1.10	0.308
Lung cancer	17 (10.2%)	1.33	13 (3.3%)	1.02	0.583
Malignant lymphoma	1 (0.6%)	0.08	9 (2.3%)	0.70	0.021
Other cause	46 (27.5%)	3.59	31 (7.8%)	2.43	0.106
Unknown origin	6 (3.6%)	0.46	7 (1.8%)	0.55	1.000

†Data are number of patients (%) and development rates of each malignancy per 1000 person years. ‡Comparison of new development in each malignancy between both groups by log rank test.

Table 3 Development rate of Each Malignancy between the non-alcoholic fatty liver disease (NAFLD) group and the hepatitis C virus (HCV) group based on the difference of gender†

Malignancies	Male		*P*‡	Female		*P*‡
	NAFLD ($n = 1200$)	HCV ($n = 1200$)		NAFLD ($n = 400$)	HCV ($n = 400$)	
Total	13.96	34.17	<0.001	10.31	20.93	<0.001
Hepatocellular carcinoma	0.83	23.75	<0.001	0.63	10.83	<0.001
Gastric cancer	2.91	2.40	0.571	1.88	1.39	1.000
Colon cancer	2.42	2.19	0.655	1.88	1.39	1.000
Lung cancer	1.33	1.05	0.676	1.25	0.93	1.000
Malignant lymphoma	0.08	0.63	0.124	0.00	0.93	0.577
Prostate cancer	1.64	1.10	0.306			
Breast cancer				1.81	1.41	1.000
Other cause	3.59	4.38	0.604	2.43	1.71	0.577
Unknown origin	0.46	0.52	1.000	0.30	0.62	1.000

†Data are development rates of each malignancy per 1000 person years. ‡Comparison of new development in each malignancy between NAFLD group and HCV group based on the difference of gender by log rank test

carcinogenesis of prostate based on obesity. Our results show that physicians in charge of NAFLD patients should pay attention to the malignancies of stomach, colon, prostate, and lung in addition to development of HCC. Moreover, aging, hyperglycemia, and smoking were dominating factors to enhance the development of malignancies in NAFLD group.

Second, HCC in the HCV group accounted for about two-thirds of the outbreak of malignancies. In the present study, the development rates of HCC and malignant lymphoma in the HCV group were statistically higher than those in the NAFLD group. The high incidences of HCC and malignant lymphoma have been reported by many reseachers.[15–19,34] Male, hyperglycemia, elevated AST, hypoalbuminemia, thrombocytopenia, and elevated AFP were dominating factors to enhance the development of malignancies in the HCV group. Hypoalbuminemia, thrombocytopenia,

Table 4 Predictive factors for malignancies in the non-alcoholic fatty liver disease (NAFLD) group†

Variables	Univariate analysis		Cox-regression	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age (years, ≥70/<70)	2.34 (1.60–3.44)	<0.001	2.09 (1.42–3.07)	<0.001
Gender (M/F)	1.11 (0.76–1.60)	0.631		
BMI (≥25/<25)	0.74 (0.52–1.04)	0.079		
Hypertension (−/+)	1.27 (0.88–1.84)	0.197		
Smoking (+/−)	1.62 (1.18–2.24)	0.003	1.64 (1.18–2.27)	0.003
AST (IU/L, ≥34/<34)	1.03 (0.62–1.70)	0.973		
ALT (IU/L, ≥36/<36)	1.27 (0.76–2.08)	0.357		
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, ≥150/<150)	1.20 (0.85–1.69)	0.282		
Total cholesterol (mg/dL, ≥220/<220)	1.39 (0.87–2.23)	0.170		
Glucose (DM/ pre-DM/non-DM)	1.39 (1.14–1.69)	0.001	1.32 (1.08–1.61)	0.007
Platelet ($\times 10^4/mm^3$, <15/≥15)	1.41 (1.02–1.96)	0.036		
AFP (ng/mL, ≥10/<10)	1.11 (0.35–3.48)	0.338		

†Data are number of patients or mean ± standard deviation.
AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; DM, diabetes mellitus, FPG, fasting plasma glucose; GGT, gamma-glutamyltransferase.

Table 5 Predictive factors for malignancies in the hepatitis C virus (HCV) group†

Variables	Univariate analysis		Cox-regression	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age (years, ≥70/<70)	1.41 (1.11–1.78)	0.003		
Gender (M/F)	1.78 (1.4692.10)	<0.001	1.49 (1.16–1.94)	0.002
BMI (≥25/<25)	1.85 (0.71–4.85)	0.201		
Hypertension (+/−)	1.20 (1.01–1.44)	0.045		
Smoking (+/−)	1.71 (1.43–2.10)	<0.001		
AST (IU/L, ≥36/<36)	2.26 (1.73–3.01)	<0.001	1.75 (1.13–2.70)	0.010
ALT (IU/L, ≥30/<30)	1.69 (1.33–2.16)	<0.001		
GGT (IU/L, ≥109/<109)	1.99 (1.53–2.58)	0.014		
Albumin (g/dL, <3.9/≥3.9)	2.07 (1.65–2.56)	<0.001	1.51 (1.15–1.97)	0.002
Triglyceride (mg/dL, ≥150/<150)	1.15 (0.56–2.41)	0.789		
Total cholesterol (mg/dL, ≥220/<220)	0.51 (0.19–1.35)	0.159		
Glucose (DM/pre-DM/non-DM)	1.37 (1.23–1.55)	<0.001	1.35 (1.18–1.59)	<0.001
Platelet ($\times 10^4/mm^3$, <15/≥15)	2.28 (1.81–2.92)	<0.001	1.49 (1.14–1.96)	0.002
AFP (ng/mL, ≥10/<10)	3.10 (2.46–4.11)	<0.001	2.50 (1.94–3.44)	<0.001

†Data are number of patients or mean ± standard deviation.
AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; DM, diabetes mellitus, GGT, gamma-glutamyltransferase.

and elevated AFP indicate the advanced liver fibrosis: it is probable that these factors enhance the HCC development as reported before.[35] Our result shows that HCV positive males with hyperglycemia, hypoalbuminemia, elevated AST, thrombocytopenia, and elevated AFP should be carefully checked for HCC.

Third, there were no significant differences in the development of each malignancy between males and females in the NAFLD group. On the other hand, rare development of HCC in males was statistically higher than that of females. However, there are no significant differences in the development of each malignancy except for HCC between males and females in the HCV group. This result suggests that development differences based on gender except for HCC in HCV group might be not important.

Cirrhotic NASH enhances the liver-related events such as HCC and liver failure. However, most patients with NAFLD do not have NASH. According to Japanese annual health check reports, 9–30% of Japanese adults demonstrate evidence of NAFLD by US. Since it is known that about 10% of individuals with NAFLD have NASH, the prevalence of NASH is estimated to be 1–3% of the adult Japanese population.[14] In patients with cirrhotic NASH, HCC and liver failure are the main causes of morbidity and mortality (5-year cumulative HCC development rate 11.3%, 5-year survival rate 75.2%, respectively). However, in the present study, most NAFLD was thought to be non-NASH. Our results suggest that patients with NAFLD before progression to NASH should be followed up to closely check the malignancies other than HCC in addition to HCC. On the other hand, patients with HCV should be followed up to take care to check liver-related disease containing HCC

The present study was limited that most of the NAFLD patients were not undergoing histological or morphological assessment by peritoneoscopy or liver biopsy before the starting time of follow up owing to their advanced age on the day of the first consulting or normal transaminase. Another limitation was that there are several differences in clinical background such as liver fibrosis between the NAFLD and HCV groups. This heterogeneity makes it slightly difficult to interpret the results of the study. On the other hand, the strengths of the present study are a long-term follow-up with a large number of patients included.

Our results indicate the following: (i) Physicians in charge of NAFLD patients should pay attention to the carcinogenesis development of stomach, colon, prostate, and lung containing HCC; and (ii) physicians in charge of HCV patients should closely check for HCC.

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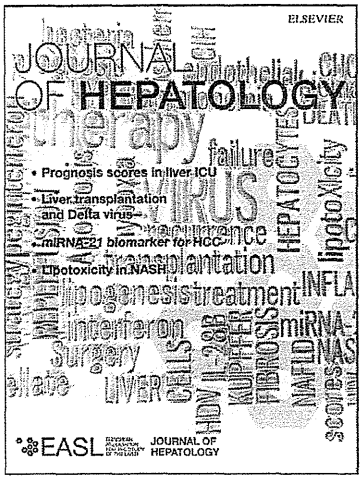

Characterization of virologic escape in hepatitis C virus genotype 1b patients treated with the direct-acting antivirals daclatasvir and asunaprevir

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

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

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Abstract

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

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

Results: There were three viral breakthroughs and four relapsers. Baseline NS3 polymorphisms (T54S, Q80L, V170M) at amino acid positions previously associated with low-level resistance (<9-fold) to select NS3 protease inhibitors were detected in four null-responders and three ineligibles but were not associated with virologic failure. Baseline NS5A polymorphisms (L28M, L31M, Y93H) associated with daclatasvir resistance (<25-fold) were detected in five null-responders and six ineligibles. All three viral breakthroughs and 2/4 relapsers carried a baseline NS5A-Y93H polymorphism. NS3 and NS5A resistance-associated variants were detected together (NS3-D168A/V,NS5A-L31M/V-Y93H) after virologic failure. Generally, daclatasvir-resistant substitutions persisted through 48 weeks

post-treatment whereas asunaprevir-resistant substitutions were no longer detectable. Overall, 5/10 patients with baseline NS5A-Y93H experienced virologic failure while 5/10 achieved a sustained virologic response.

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

Abstract word count: 250 words

Keywords: asunaprevir, daclatasvir, drug resistance, direct-acting antivirals, hepatitis C, peginterferon-sparing.

Introduction

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

Daclatasvir (DCV) and asunaprevir (ASV) are currently undergoing clinical development for HCV infection. DCV (BMS-790052) is a first-in-class, highly selective NS5A replication complex inhibitor with picomolar potency and broad HCV genotypic coverage [6] that has demonstrated antiviral efficacy and good tolerability in combination with alfa/RBV [7]. ASV (BMS-650032) is a selective inhibitor of NS3 protease with antiviral activity *in vitro* against GT1 and GT4 [8]; it has also been shown to be efficacious and generally well tolerated in combination with alfa/RBV [9]. Clinical interest is increasingly focusing on exploring DAA-only regimens without alfa/RBV, whose potential benefits might include better tolerability and compliance, and a reduced duration of therapy. One recent alfa/RBV-sparing study of DCV plus ASV (AI447017) has examined the efficacy and safety of this combination for 24 weeks in a small cohort of ten GT1b null-responders, in which an SVR rate of 90% was

observed [10]. The study was then expanded to include an additional cohort of null-responders and a group of patients ineligible to receive, or intolerant of, alfa/RBV [11].

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

Patients and methods

Study design and patients

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

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

The full study design, including inclusion/exclusion criteria, and safety/efficacy endpoints, is described elsewhere [11]. Briefly, eligible patients were men and women aged 20–75 years with HCV genotype 1 infection ≥6 months and HCV RNA $\geq 10^5$ IU/mL. Patients were excluded if they had evidence of liver cirrhosis within 24 months of screening; a history of

hepatocellular carcinoma, other chronic liver disease, variceal bleeding, hepatic encephalopathy, or ascites requiring diuretics or paracentesis; coinfection with hepatitis B virus or HIV; or other clinically significant medical conditions.

Laboratory assessments

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

Genotypic and phenotypic analysis of clinical samples

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

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

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

Results

Viral response to DCV and ASV

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

Null-responders

Virologic response

Rapid and similar decreases in plasma HCV-RNA levels were observed among patients who initiated treatment with ASV 600mg (Fig. 1A) or ASV 200mg (Fig. 1B). Mean reduction in HCV-RNA at Week 1 was comparable for both groups (–4.4 versus –4.3 $\log_{10}$ IU/mL,

respectively). Of the patients still receiving treatment (P-6 discontinued at Day 16 due to an AE), all but one patient (P-13) had HCV-RNA <15 IU/mL at Week 4 and 52% had undetectable HCV-RNA at this time.

Baseline analysis

Baseline *IL28B* genotype and naturally occurring polymorphisms associated with ASV or DCV resistance (resistance-associated variants [RAVs]) are shown in Table 1. As anticipated for this prior null-responder population, the majority (18/21) were non-CC *IL28B*. The NS5A polymorphism Y93H (24-fold DCV resistance [13]) was observed in three patients. Other polymorphisms conferring minimal (2- to 3-fold) DCV resistance were detected in two patients (NS5A-L28M-R30Q and NS5A-L31M). Polymorphisms associated with minimal to low-level resistance to select NS3 protease inhibitors (one patient, NS3-T54S-Q80L; one patient, NS3-Q80L-V170I/M; two patients, NS3-Q80L) [4, 5, 18] were also observed.

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