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Alcohol consumption and recurrence of non-B or non-C hepatocellular carcinoma after hepatectomy: a propensity score analysis

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Abstract
Background The aim of this study was to identify factors related to the recurrence of non-B or non-C (NBNC) hepatocellular carcinoma (HCC).
Study design Between April 2000 and March 2012, out of 621 consecutive HCC patients at our institution, 543 who underwent initial hepatectomy and had no extrahepatic metastases were enrolled in the study. Multivariate analysis were performed to identify risk factors for poor disease-free survival (DFS).
Results The 5-year DFS rate of NBNC (34 %) was better than that of hepatitis virus B (30 %, $P = 0.011$) and hepatitis virus C (21 %, $P < 0.0001$), significantly. Multivariate analysis revealed NBNC [hazard ratio (HR), 0.5; 95 % CI, 0.4–0.8; $P < 0.0001$)] to be an independent factor for DFS rate. We constructed a propensity score matching model with the 543 patients, and the 5-year DFS rates with and without severe alcohol liver disease (ALD) were 31.6 and 47.5 %, respectively ($P = 0.013$). In the 163 NBNC patients, severe ALD, mild ALD, and no ALD were seen in 35, 56, and 72 patients, respectively. Multivariate analysis revealed a vascular invasion into the hepatic vein (HR, 3.3; 95 % CI, 1.7–6.3; $P < 0.0001$) and severe ALD (HR, 2.0; 95 % CI, 1.1–3.6; $P = 0.020$) to be independent risk factors for poor DFS. By propensity score matching between mild and severe ALD, the 5-year DFS rates with severe and mild ALD were 26 and 50 %, respectively ($P = 0.035$).
Conclusions The prognoses of NBNC patients were better than those of patients with viral infections. Among the NBNC patients, preoperative excessive alcohol intake decreased DFS rate of HCC occurrence after surgery.

Keywords Hepatitis B virus · Hepatitis C virus · Non-B non-C · Hepatocellular carcinoma · Recurrence · Hepatectomy

Abbreviations

AFP	Alpha-fetoprotein
DCP	Des-gamma-carboxy prothrombin
DFS	Disease-free survival
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
NBNC	Non-B non-C
HR	Hazard ratio
OS	Overall survival

Introduction

Primary liver cancer involving hepatocellular carcinoma (HCC) is the fifth most common and fatal cancer worldwide. HCC has been the most rapidly increasing cancer-related cause of death in developed countries including Japan, Australia, Canada, the United States, and throughout Europe over the last two decades. The number of non-B non-C (NBNC) HCC patients has increased rapidly [1]. Chronic viral hepatitis and liver cirrhosis following

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hepatitis B virus (HBV) and hepatitis C virus (HCV) infection are responsible for most HCCs. The oncogenic mechanism and clinicopathological characteristics of HCCs critically depend on the type of hepatitis virus involved [2, 3]. Patients with HBV-related HCCs may have a better liver function reserve than those with HCV-related tumors. The etiology is unclear in the other 15–50 % of new HCC cases. In Japan, 10 % of patients diagnosed with HCC have NBNC HCC.

Most patients with NBNC HCC have alcoholic liver disease or nonalcoholic fatty liver disease (NAFLD) including nonalcoholic steatohepatitis (NASH). Recent studies have indicated that both NASH and excessive alcohol intake increase the risk of developing HCC [4]. The prevalence of NAFLD and NASH is reported to be 20 %and 1 %, respectively, among adults in Japan [5, 6], and longitudinal outcome studies have reported that the prevalence of HCC in patients with NAFLD and NASH is 0–0.5 and 0–2.8 %, respectively, over a period of up to 19.5 years [7–10]. In Europe, alcohol-induced cirrhosis accounts for one-third to one-half of all HCC cases [11–13]. HCC is found in 10.1 % of patients with cirrhosis caused by alcohol alone, and its prevalence is almost identical to that of HCV infection [14]. The risk of developing HCC increases when daily alcohol consumption exceeds 80 g/day, whereas the adjusted odds ratio is not increased significantly for patients who consume alcohol at less than 80 g/day [15]. However, some reports indicate that patients with NBNC HCC present with more advanced tumors with poor differentiation, invasion, and vascular involvement and a higher incidence of intrahepatic metastases than patients with HCCs associated only with viral infection [16–18].

In this study, we aimed to elucidate the clinicopathological features of patients with NBNC HCC who had undergone hepatectomy, and the factors, including preoperative alcoholism, that are associated with recurrence. For a fair comparison, key factors that were responsible for DFS were adjusted for by using propensity score-matched analysis. Moreover, we examined whether alcoholism promotes the recurrence of HCC after hepatectomy and whether preoperative alcohol consumption is the best predictor of DFS in patients with NBNC HCC.

Patients and methods

Between April 2000 and March 2012, a total of 621 patients received initial treatment for HCC at the Department of Hepatobiliary Pancreatic Surgery, Tokyo Medical and Dental University. Of these patients, 545 patients underwent initial hepatectomy for HCC and were not found to have extrahepatic metastases. The 543 patients, excluding two (one with autoimmune hepatitis and one with primary biliary cirrhosis), were enrolled in the unadjusted study. The baseline characteristics of the patients are shown in Table 1 (The data of four HBV + HCV patients are not shown). We classified NBNC patients into severe ALD group (alcohol consumption $\geq$ 80 g/day), no ALD (alcohol consumption $<$ 20 g/day), and mild ALD group (20 g/day $\leq$ alcohol consumption $<$ 80 g/day). Alcoholic history was available in 463 patients. Occult HBV infection is defined by the absence of serologically detectable HBs antigen despite the presence of HBc antibody in serum [19, 20].

The decision to perform hepatic resection with anatomical resection is generally determined by the Child-Pugh A/B score and the indocyanine green retention rate at 15 min (ICG-R15) according to the Makuuchi criteria. Non-anatomic resection includes partial resection. In the anatomic resections performed in our study, the liver was divided along the demarcation line after occlusion of the portal vein and hepatic artery. When necessary, the main feeding artery was identified by intravenous injection of sonazoid [21]. We divided the liver parenchyma using an ultrasonic dissector and other energy devices. Prior to resection, all tumors were examined by intraoperative ultrasonography and preoperative computed tomography (CT). Intraoperative ultrasonography with contrast enhancement was used, if necessary [22]. The size of the tumors and length of the surgical margin were measured before fixation of the specimens. The extent of macrovascular invasion was determined using preoperative CT, as microvascular invasion could not be determined before hepatectomy. Microvascular invasion was evaluated on the basis of histological findings if macrovascular invasion was not noted. Background liver cirrhosis and surgical margins were assessed by microscopic examination of the specimens. After discharge, all the patients were examined for recurrence by ultrasonography every 3 months and by dynamic CT every 6 months. The median follow-up period after surgery was 2.9 years (range 0–11.2 years). DFS was defined as the interval between the operation and the date on which recurrence was diagnosed or the end of the observation period if no recurrence was noted. The general rules for the clinical and pathological study of primary liver cancer by liver cancer study group of Japan (5th edition, revised version) simply classify the liver histology into normal liver, chronic hepatitis, and liver cirrhosis. The rule describes the classification of the hepatic fibrosis in detail, as follows: no fibrosis (f0), increased fibrosis of portal area (f1), bridging fibrosis (f2), bridging fibrosis with distorted hepatic lobules (f3), and liver cirrhosis (f4). The patients' medical records were reviewed systematically for relevant clinical data (gender, age, viral infection, alcohol use, and liver function), tumor factors (primary tumor size and

Table 1 Baseline characteristics of patients with non-B non-C hepatocellular carcinoma

	HBV ($N = 96$)	HCV ($N = 275$)	NBNC ($N = 168$)	P
Age (years)	59.3 ± 11.4	68.4 ± 7.6	68.5 ± 11.2	<0.0001*
Gender				
Male	74 (77 %)	200 (73 %)	141 (84 %)	0.025*
Alcoholism (+)	21 (25 %)	66 (26 %)	100 (60 %)	<0.0001*
Severe ALD (+)	9 (12 %)	18 (8 %)	35 (24 %)	<0.0001*
Liver function				
ICG-R15 (%)	15.1 ± 11.7	19.3 ± 11.4	15.2 ± 9.5	<0.0001*
AST (IU/L)	48.7 ± 45.8	60.3 ± 41.7	42.6 ± 27.0	<0.0001*
Platelet (10^4/mL)	16.2 ± 8.1	13.5 ± 6.7	19.5 ± 11.0	<0.0001*
Prothrombin time (%)	84.5 ± 18.2	85.9 ± 15.3	86.1 ± 16.6	0.853
Albumin (g/dL)	4.0 ± 0.5	3.8 ± 0.6	4.0 ± 0.4	<0.0001*
Total bilirubin (mg/dL)	1.0 ± 1.0	0.8 ± 0.4	0.9 ± 0.5	0.443
Child Pugh score	4.9 ± 1.6	5.2 ± 1.3	4.8 ± 1.7	0.092
Tumor factors				
Tumor size (cm)	5.4 ± 4.4	4.0 ± 2.5	5.8 ± 4.1	<0.0001*
Tumor number	1.5 ± 1.1	1.6 ± 1.0	1.5 ± 1.2	0.98
Alpha-fetoprotein (ng/mL)	12854 ± 66264	3497 ± 27261	2477 ± 14500	0.179
DCP (AU/L)	6267 ± 31637	3351 ± 17435	11644 ± 44165	0.101
Anatomic resection (+)	68 (71 %)	162 (59 %)	123 (73 %)	0.017*
Pathological findings				
Micro-vascular invasion				
vp (+)	49 (51 %)	101 (37 %)	69 (41 %)	0.102
vv (+)	12 (13 %)	33 (12 %)	22 (15 %)	0.829
b (+)	5 (5 %)	13 (5 %)	15 (9 %)	0.188
Chronic hepatitis (+)	35 (36 %)	112 (41 %)	65 (40 %)	0.793
Liver cirrhosis (+)	50 (52 %)	153 (56 %)	55 (34 %)	<0.0001*
Surgical margin (+)	19 (20 %)	53 (19 %)	23 (14 %)	0.270

Values are shown as the mean ± SD

ALD alcoholic disease, *DCP* des-gamma-carboxy prothrombin, *HBV* hepatitis B virus, *HCV* hepatitis C virus, *NBNC* non-HBV non-HCV

* $P < 0.05$ considered statistically significant

tumor markers), operative procedure, and pathological findings. We determined alcoholism as some mental and/or physical status related to alcohol dependence [23]. Follow-up data were updated yearly or at shorter intervals, and the last follow-up examination was performed in March 2012.

Statistical analysis were performed using SPSS version 21.0 (IBM Inc., Chicago, IL, USA), unless otherwise stated. Analysis of variance and the χ^2 test were used for continuous and categorical data, respectively. The odds ratio for recurrence for each factor was examined by univariate analysis using the Cox proportional hazards model. Variables found to be statistically significant on this basis were entered into multivariate analysis. DFS was analyzed using the Kaplan–Meier method and the log-rank test. A P value of <0.05 was considered statistically significant; all tests were two-sided.

Because hepatectomy was not performed on the basis of random assignment in the present study, confounding factors could hamper the observations obtained from unadjusted factors. To reduce the potential bias, a propensity score [24] was calculated to assess the conditional probability of treatment according to the individual's covariates and to balance treatment choice-related variables such that the analysis simulated random assignment [25].

The propensity score was estimated using a logistic regression model in which outcome was the binary variable, severe ALD group versus mild ALD group (0, mild ALD groups; 1, severe ALD group), and the explanatory variables were the independent factors obtained from the multivariate analysis for DFS, such as pathological chronic hepatitis, preoperative serum albumin level, and tumor vascular invasion into the hepatic vein in the analysis of 91 NBNC patients. The propensity score was estimated between the categorizing of severe ALD group versus no-severe ALD group (0, no-severe ALD groups; 1, severe

ALD group) in the analysis of 543 patients. Without replacement, one-to-one pair matching by estimated propensity score generated 15 matched pairs of mild versus severe ALD (Table 4) and 55 matched pairs of patients with and without severe ALD (Supplementary Table 2). All matching processes were performed by the aforementioned SPSS version 21.0. The degree of covariate imbalance in the unmatched and matched samples was measured using the standardized (mean and proportion) difference proposed by Austin et al [26]. It has been suggested that a standardized difference of greater than 20 % represents meaningful imbalance in a given variable between treatment groups [27].

Results

The baseline characteristics of the patients are summarized in Table 1. Liver function of the NBNC patients was, on average, better than that of HCV-infected patients, as judged by the ICG-R15, aspartate aminotransferase level, platelet count, and albumin level ($P < 0.0001$); while the prothrombin time, total bilirubin level, and Child-Pugh scores were comparable. Liver cirrhosis was significantly less frequent in NBNC patients (34 %) than in HCV- (56 %) and HBV- (52 %) infected patients ($P < 0.0001$). The mean tumor size was larger in the NBNC patients than in the HCV-infected patients, whereas the other indices of tumor malignancy, such as microvascular invasion, serum alpha-fetoprotein (AFP) level, and des-gamma-carboxy prothrombin (DCP) level, did not vary significantly. Alcoholism and severe ALD were more evident in NBNC patients than in the other two groups ($P < 0.0001$). The groups did not differ significantly with respect to the pathological surgical margin, although it was noteworthy that non-anatomic resection was frequently selected for HCV-infected patients. As shown in Fig. 1, the DFS rate of NBNC patients was longer than that of HBV and HCV patients. The 5-year DFS rates were 30, 21, and 34 % in the HBV, HCV, and NBNC groups, respectively. The NBNC patients experienced recurrence less frequently than did patients infected with HBV ($P = 0.011$) and HCV ($P < 0.0001$; Fig. 1). We excluded the cases of autoimmune hepatitis and primary biliary cirrhosis from NBNC group.

In univariate analysis of 543 patients, NBNC was an important determinant for good prognosis (HR, 0.6; 95 % CI, 0.4–0.8, $P < 0.0001$), as shown in Supplemental Table 1. The other determinants were liver functional reserve factors (ICG-R15, serum AST, prothrombin time, and serum albumin), tumor factors (size, number, serum tumor marker, vascular invasion), noncancerous liver histology (chronic liver hepatitis and liver cirrhosis), and

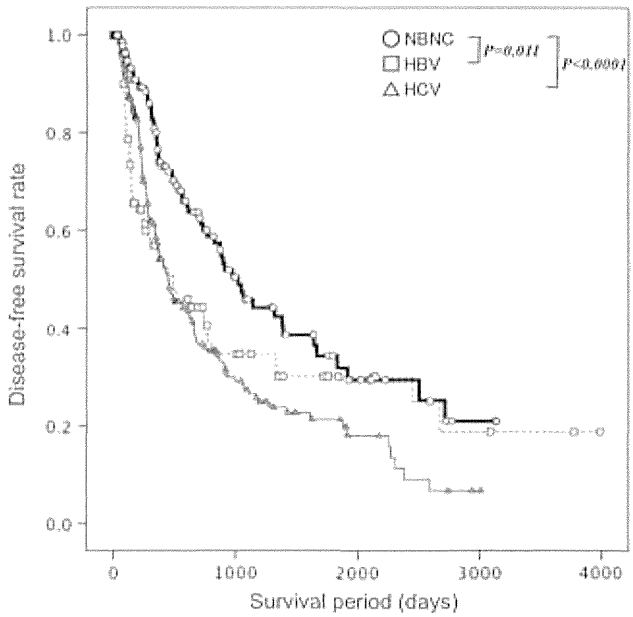

Fig. 1 Disease-free survival of patients with non-B non-C (NBNC) hepatocellular carcinoma. *Open squares*, *triangles*, and *circles* denote the disease-free survival (DFS) of patients with HBV, HCV, and NBNC, respectively. The DFS of the non-B non-C (NBNC) group was better than that of the HBV group ($P = 0.011$) and HCV group ($P < 0.0001$)

surgical factors (anatomic resection, surgical margin). Multivariate analysis revealed that NBNC (HR, 0.5; 95 % CI, 0.4–0.8, $P < 0.0001$), ICG-R15, serum AST, tumor number, vascular invasion, anatomic resection and pathological chronic hepatitis. Taking into account factors related to prognosis, we compared the DFS rate of patients in the presence and absence of the severe ALD, adjusting for the risk factors using propensity score matching. The area under the ROC curves (C value) was 0.892 ± 0.016 SE for predicting severe ALD considering alcoholism. As shown in Supplemental Table 2, all factors related to recurrence were adjusted significantly considering the propensity score constructed with the aforementioned factors. There was no significant difference between the two groups with respect to propensity score after the adjustment ($P = 1.000$), though there was a significant difference before the propensity adjusting ($P < 0.0001$). Younger age, male gender, alcoholism, and higher albumin level observed in the severe ALD group before the matching were completely adjusted after the matching. The DFS rates with and without the severe ALD groups were compared (Supplemental Fig. 1). The 1-, 3-, and 5-year DFS rates were 70, 32, and 32 % in the severe ALD group and 76, 68, and 48 % in the no-severe ALD group, respectively. There was a remarkable difference between the two groups with respect to DFS rates (log-rank; $P = 0.013$). These results suggest that severe

ALD also increases the risk of HCC recurrence amongst all patients with NBNC HCC.

These findings led us to determine which factor decides the DFS rate of NBNC patients. A total of 35 out of 168 NBNC patients were classified as having severe ALD (Table 2). The alcoholic history was available in 163 NBNC patients. Of these 168 patients, 17 patients tested positive for serum HBc antibody (+). Severe ALD was associated with being male ($P = 0.005$), alcoholism ($P < 0.0001$), small tumor size ($P = 0.040$) and liver cirrhosis (f4) ($P = 0.011$). There was no difference in all fibrosis grades except for liver cirrhosis grade (f4) among the three groups. As shown, there was no difference in fibrosis grade (f0–3) among the three groups. There was no significant difference among the groups with respect to any of the other factors. The mean follow-up period after surgery was 2.7 years. As shown in Fig. 2, the 5-year DFS rates were 25.2 and 51.2 % in the severe and mild ALD, respectively ($P = 0.013$). However, the result may be biased by additional determinants of DFS, for example, liver cirrhosis. Liver cirrhosis (f4) was the most evident in the severe ALD group among the three groups, though the liver function was not different and tumor size was the largest in the no-ALD group.

Table 3 summarizes the results of univariate analysis of DFS in 163 NBNC patients (excluding five patients whose alcohol histories were not available), which show that a decreased serum albumin level ($P = 0.033$), tumor number

Table 2 Background characteristics of 168 NBNC patients with alcohol consumption

	Non-B non-C hepatocellular carcinoma			
	Severe ALD ($n = 35$) Mean ± SD	Mild ALD ($n = 56$) Mean ± SD	No ALD ($n = 72$) Mean ± SD	*P*
Age (years)	65.3 ± 8.6	69.2 ± 9.7	69.4 ± 13.2	0.166
Gender				
Male	33/94 %	51/91 %	53/74 %	0.005*
AST (IU/L)	45.8 ± 32.0	40.6 ± 26.9	42.8 ± 24.5	0.677
Platelet ($10^4/\mu L$)	17.4 ± 7.5	19.1 ± 9.6	20.7 ± 13.0	0.337
Alcoholism (+)	28/90 %	42/75 %	5/7 %	<0.0001*
HBc antibody (+)	2/6 %	2/4 %	13/22 %	0.017*
Liver function				
ICG-R15 (%)	14.7 ± 8.3	15.9 ± 9.5	15.1 ± 10.3	0.810
Prothrombin time (%)	87.6 ± 12.5	84.7 ± 20.3	86.1 ± 15.4	0.919
Albumin (g/dL)	4.2 ± 0.3	4.0 ± 0.4	4.0 ± 0.5	0.107
Total bilirubin (mg/dL)	0.9 ± 0.4	0.9 ± 0.6	0.9 ± 0.5	0.767
Child-Pugh score	4.4 ± 1.8	4.8 ± 1.6	5.0 ± 1.6	0.175
Tumor factors				
Tumor size (cm)	4.9 ± 3.1	5.2 ± 3.8	6.7 ± 4.5	0.040*
Number	1.5 ± 0.7	1.6 ± 1.7	1.4 ± 1.0	0.611
Alpha-fetoprotein (ng/mL)	2357 ± 8358	435 ± 1758	3923 ± 21024	0.420
DCP (AU/L)	2970 ± 8721	8142 ± 34595	14434 ± 44929	0.296
Anatomic resection (+)	27/77 %	35/63 %	58/81 %	0.062
Pathological findings				
Surgical margin (+)	3/9 %	9/16 %	11/15 %	0.394
No fibrosis (f0) (+)	5/14 %	19/35 %	23/33 %	0.128
Portal fibrosis (f1) (+)	7/20 %	5/9 %	10/14 %	0.354
Bridging fibrosis (f2) (+)	1/3 %	8/15 %	12/17 %	0.110
Distorted lobules (f3) (+)	3/9 %	4/7 %	7/10 %	0.867
Liver cirrhosis (f4) (+)	19/54 %	18/33 %	17/25 %	0.011*
Chronic hepatitis (+)	11/31 %	19/35 %	34/49 %	0.133
Micro-vascular invasion				
vp (+)	11/31 %	23/41 %	34/47 %	0.297
vv (+)	3/9 %	7/14 %	12/19 %	0.410
b (+)	5/14 %	4/7 %	6/8 %	0.489

Values are shown as the mean ± SD. The alcohol history was not available in five patients

b biliary invasion, *DCP* des-gamma-carboxy prothrombin, *pv* portal venous invasion, *vv* hepatic venous invasion

* $P < 0.05$ considered significant

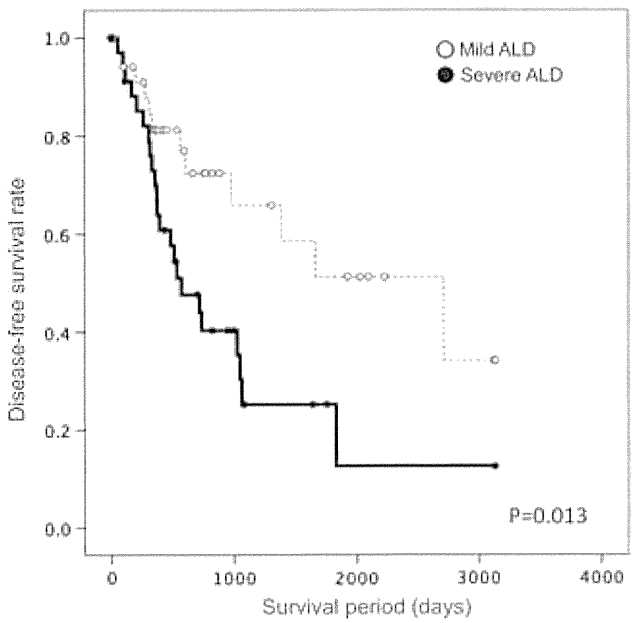

Fig. 2 Disease-free survival of non-B non-C patients in severe and mild alcohol liver disease (ALD) groups before adjustment with propensity scores. *Open* and *closed circles* denote the mild and severe ALD patients, respectively. The difference between the two groups was remarkable ($P = 0.013$)

($P = 0.034$), an elevated serum AFP level ($P = 0.004$), vascular invasion into the hepatic vein ($P = 0.001$), and severe ALD ($P = 0.02$) were possible risk factors. Occult HBV infection was more frequently found in no-ALD patients, although this did not reach statistical significance ($P = 0.223$). Multivariate analysis revealed that vascular invasion into the hepatic vein (HR, 3.3; 95 % CI, 1.7–6.3; $P \leq 0.0001$) and severe ALD (HR, 2.0; 95 % CI, 1.1–3.6; $P = 0.020$) were also risk factors for DFS.

To make a fair comparison, taking into account alcohol consumption as a factor related to prognosis, we adjusted for the risk factors using propensity score matching. As shown in Table 4, all factors related to recurrence were adjusted significantly. There was no significant difference between the two groups with respect to propensity score ($P = 1.000$). For the risk factors examined, we found that standard difference, an index for the imbalance between sample groups, significantly improved from beyond 20 % before adjustment with propensity score matching to within 20 % after adjustment (data not shown). The standardized difference of propensity score before matching (70.9 %) was significantly adjusted after matching (0 %). After adjusting the score, the DFS rates of the severe and mild ALD groups were compared (Fig. 3). The 1-, 3-, and 5-year DFS rates were 84, 64, and 50 % in the mild ALD group and 69, 42, and 26 % in the severe ALD group, respectively. There was a remarkable difference between the two groups with respect to DFS rates (log-rank; $P = 0.035$). These results suggest that severe ALD also increases the risk of HCC recurrence amongst NBNC patients.

Discussion

The present study suggests that preoperative severe ALD increases the risk of HCC recurrence after hepatectomy in HCC patients involving NBNC-derived HCC (Fig. 3, Supplemental Fig. 1) and the DFS rate in patients with NBNC-related HCC was superior to that in patients with HCV- or HBV-related HCC (Fig. 1, Supplemental Table 1). Propensity score matching allowed a fair comparison of the severe ALD and the other groups, as shown in Table 4 and Supplemental Table 2.

In NBNC patients, all of the factors tested for an association with HCC recurrence by multivariate analysis were also adjusted (Table 4). Propensity score in the severe and mild ALD groups were comparable after the matching ($P = 1.000$), though the propensity score value in the severe ALD group was significantly higher than that in the mild ALD group before the matching ($P = 0.002$). Before adjusting for the confounding factors by the matching, we found that being male, alcoholism, relatively small tumor size, and liver cirrhosis were all significantly more common in the severe ALD group (Table 2). As shown in Fig. 2, the 5-year DFS rates in the severe and mild ALD groups were 25 and 51 %, respectively ($P = 0.013$). After adjusting for the prognostic indices (Fig. 3), the difference in the DFS rate between patients who did and did not show severe ALD was not changed (26 vs. 50 %, $P = 0.035$). In NBNC patients, using propensity score matching, we came to this conclusion because the *C*-value to estimate how the score would predict the severe ALD patients was 67 % (95 % CI, 56–78.3 %; $P = 0.007$) (data not shown).

In 543 patients (including HBV, HCV, and NBNC patients), all of the factors tested for an association with HCC recurrence by multivariate analysis were also adjusted (Supplemental Table 2). Propensity scores in the presence and absence of severe ALD were comparable after the matching ($P = 1.000$), though the propensity score in the severe ALD group was significantly higher before the matching ($P < 0.0001$). The C-value of the score estimating the severe ALD patients was 89 % (95 % CI, 86–92 %; $P < 0.0001$). Before adjusting for the confounding factors by the matching, we found that being a younger male, alcoholism, and higher serum albumin were all significantly more common in the severe ALD group (Supplemental Table 2). All factors were adjusted by the propensity score matching. After the adjusting for the prognostic indices (Supplemental Fig. 1), the DFS rates of patients who did and did not show severe ALD were 32 and 48 %, respectively ($P = 0.013$). These results suggest that

Table 3 Univariate and multivariate analysis for the disease-free survival of 168 non-B non-C patients

	Univariate analysis			Multivariate analysis		
	HR	95.0 % CI	*P*	HR	95.0 % CI	*P*
Gender						
Female	0.5	(0.2–1.1)	0.100			
Age (years)						
>71	0.8	(0.5–1.3)	0.327			
Severe ALD (+)	1.8	(1.1–3.1)	0.021*	2.0	(1.1–3.6)	0.020*
Alcoholism (+)	1.1	(0.6–1.7)	0.809			
HBcAb (+)	1.8	(0.7–4.4)	0.223			
Liver functional factor						
ICG-R15 (%)						
>13	1.2	(0.7–2.0)	0.433			
AST (IU/L)						
>34	1.3	(0.8–2.1)	0.317			
Platelet (10^4/μL)						
>17.8	1.4	(0.9–2.3)	0.164			
Prothrombin time (%)						
>86.1	0.8	(0.5–1.4)	0.484			
Albumin (g/dL)						
>4.1	0.6	(0.3–1.0)	0.033*	0.6	(0.4–1.1)	0.109
Total bilirubin						
>0.8	1.2	(0.7–1.9)	0.546			
Child-Pugh score						
>6	1.8	(1.0–3.3)	0.057			
Tumor factor						
Tumor size (cm)						
>5	1.2	(0.7–2.0)	0.543			
Multiple (+)	1.8	(1.0–3.0)	0.034*	1.4	(0.8–2.5)	0.218
AFP (ng/mL)						
>8	2.1	(1.3–3.6)	0.004*	1.6	(0.9–2.7)	0.121
DCP (AU/L)						
>75	1.6	(1.0–2.6)	0.074			
Anatomic resection (+)	0.8	(0.5–1.4)	0.472			
Pathological findings						
Micro-vascular invasion						
vp (+)	1.3	(0.8–2.2)	0.235			
vv (+)	2.9	(1.6–5.4)	0.001*	3.3	(1.7–6.3)	<0.0001*
b (+)	1.7	(0.8–3.8)	0.182			
Surgical margin (+)	1.7	(0.9–3.2)	0.124			
Chronic hepatitis (+)	0.6	(0.3–1.1)	0.088			
Liver cirrhosis (+)	1.3	(0.7–2.3)	0.479			

The alcohol history was not available in five patients

AFP alpha-fetoprotein, *b* biliary invasion, *DCP* des-gamma-carboxy prothrombin, *vp* portal venous invasion, *vv* hepatic venous invasion

* $P < 0.05$ considered significant

severe ALD was a determinant of DFS in those HCC patients.

The present study involved 72 no-ALD patients out of 543 HCC patients (13.3 %), and 21 % of the NBNC patients were diagnosed as having severe ALD (Table 2). The prevalence of NAFLD is reported to be 20 % in Japan with or without HCC [5, 6]. HCCs are found in 10.1 % of patients with alcohol-induced cirrhosis, whereas HCCs were identified in 14–19 % of patients without cirrhosis in Western countries [28–30]. In a Japanese nationwide study with 54,003 HCC patients, 9,307 patients were classified as having NBNC HCC (17.3 %) and 35 % of them were diagnosed with severe alcoholic disease (more than 86 g/day) [31]. The ratios are higher than the present study. Multivariate analysis in the present study revealed that the severe ALD and tumor invasion into the hepatic vein that

Table 4 Baseline characteristics after the adjustment by propensity score matching in severe and mild ALD patients

	Pre-propensity Score Matching ($N = 91$)			Post-propensity Score Matching ($N = 30$)		
	Severe ALD Mean ± SD	Mild ALD Mean ± SD	P	Severe ALD Mean ± SD	Mild ALD Mean ± SD	P
Propensity score	0.45 ± 0.16	0.34 ± 0.15	0.002*	0.43 ± 0.15	0.43 ± 0.15	1.000
Age (years)	65.3 ± 8.6	69.2 ± 9.7	0.053	66.1 ± 9.0	66.0 ± 10.4	0.958
Male (+)	33/94 %	51/91 %	0.703	28/93 %	26/87 %	0.671
Alcoholism (+)	32/91 %	42/75 %	0.058	27/90 %	21/70 %	0.104
ICG-R15 (%)	14.7 ± 8.3	15.9 ± 9.5	0.515	14.6 ± 7.4	13.7 ± 8.7	0.681
AST (IU/L)	45.8 ± 32.0	40.6 ± 26.9	0.412	46.2 ± 33.9	41.1 ± 29.9	0.541
Platelet ($10^4/\mu L$)	17.4 ± 7.5	18.3 ± 9.7	0.651	17.3 ± 5.9	19.4 ± 9.6	0.312
PT (%)	87.6 ± 12.5	84.7 ± 20.3	0.464	88.1 ± 13.4	85.9 ± 19.6	0.614
Albumin (g/dL)	4.2 ± 0.3	4.0 ± 0.4	0.014*	4.1 ± 0.3	4.1 ± 0.4	0.649
T-Bil (mg/dL)	0.9 ± 0.4	0.9 ± 0.6	0.574	0.8 ± 0.4	0.9 ± 0.4	0.767
Child Pugh score	4.4 ± 1.8	4.8 ± 1.6	0.231	4.4 ± 1.8	4.6 ± 1.9	0.780
Tumor size (cm)	4.9 ± 3.1	5.2 ± 3.8	0.757	5.0 ± 3.1	5.1 ± 4.4	0.876
Tumor number	1.5 ± 0.7	1.6 ± 1.7	0.690	1.5 ± 0.7	1.5 ± 2.0	0.865
AFP (ng/mL)	2357 ± 8358	435 ± 1758	0.105	2733 ± 9018	589 ± 2341	0.228
DCP (AU/L)	2970 ± 8721	8142 ± 34594	0.396	3420 ± 9388	11084 ± 46756	0.391
Anatomic resection (+)	27/77 %	35/63 %	0.171	23/77 %	21/70 %	0.771
Pathological findings						
vp (+)	11/31 %	23/50 %	0.382	8/27 %	14/47 %	0.180
vv (+)	3/9 %	7/14 %	0.733	3/11 %	2/7 %	1.000
b (+)	5/14 %	4/7 %	0.298	5/17 %	2/7 %	0.424
Surgical margin (+)	9/9 %	9/16 %	0.359	3/10 %	5/17 %	0.706
Chronic hepatitis (+)	11/31 %	19/35 %	0.820	9/30 %	10/36 %	0.781
Liver cirrhosis (+)	19/54 %	18/33 %	0.078	17/57 %	9/32 %	0.071

Values are shown as the mean ± SD

AFP alpha-fetoprotein, *b* biliary invasion, *DCP* des-gamma-carboxy prothrombin, *PT* prothrombin time, *T-Bil* total bilirubin, *vp* portal venous invasion, *vv* hepatic venous invasion

* $P < 0.05$ considered significant

were typicall sufficient in predicting the prognosis of conventional HCC patients with viral infections were also the independent risk factors in NBNC patients. Alcoholism was not found to be a risk factor for the recurrence of HCC in NBNC patients (Table 3). The 5-year DFS rates were 30, 21, and 25 % in the HBV, HCV, and severe ALD groups of the NBNC patients, respectively (Figs. 1, 2), though the 5-year DFS rate was 51 % in the mild ALD group of NBNC patients. The malignant potential of the severe ALD group of NBNC patients may be comparable to that of HBV and HCV patients. Chronic alcohol use in the absence of viral infection significantly increased the risk of HCC by 1.6- to 4-fold when alcohol intake was defined only as drinking, without reference to the amount or frequency of alcohol consumption [32, 33]. The odds ratio increases 5- to 7-fold, especially in patients with an alcohol intake of more than 80 g/day for more than 10 years [34].

The mechanism of carcinogenesis is unknown and may be unique in NBNC patients. In the present study, liver cirrhosis was not found to be an independent determinant for HCC recurrence in multivariate analysis (Table 3), though it was more frequent in the severe ALD group (Table 2). The patients in this study were not exposed to the other chemical agents, such as aflatoxins and exogenous steroids that may cause HCC in NBNC patients. The development of HCC may not always depend on liver inflammation and fibrosis [35, 36]. Occult HBV infection was not associated with the poor prognosis of NBNC patients (Table 3). DFS after hepatectomy in patients with occult HBV infection was comparable with that in patients without occult HBV infection. The 5-year DFS rate in patients with occult HBV infection was 42 % (data not shown). Whether occult HBV infection is involved in NBNC-derived HCC is still controversial [37, 38]. The present study is consistent with the previous report. Liver functional factors did not determine the DFS rate in NBNC patients (Table 3), though the ICG-R15 and serum AST level determined the DFS rate of 543 patients

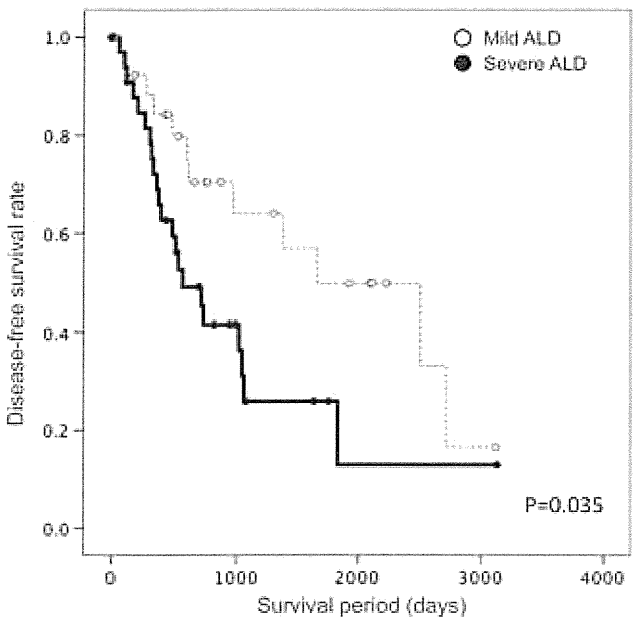

Fig. 3 Disease-free survival rates of non-B non-C patients in severe and mild alcohol liver disease (ALD) groups after adjustment with propensity scores. *Open* and *closed circles* denote the mild and severe ALD groups, respectively. The difference between the two groups was remarkable ($P = 0.035$)

(Supplemental Table 1). The liver function of NBNC patients was significantly better than that of HCV patients (Table 1). Good liver function at the initial hepatectomy may prevent early recurrence in patients with NBNC HCC without abusive alcohol consumption [2]. Such patients may have better liver function without the chronic active inflammation seen in HBV- or HCV-infected patients [39, 40].

Limitations of the present study include that the data of genome-wide gene expression and the data of urinary constituents were not available to elucidate the mechanisms of carcinogenesis in NBNC livers in the presence or absence of severe ALD. Multicentric occurrence of HCC is also associated with reduced levels of sirtuin 3, a protein that regulates hepatocellular orotic acid concentration and inhibits hepatic carcinogenesis [41, 42]. Genome-wide gene expression analysis of liver samples indicated that the multicentric occurrence of HCC was associated with decreased *SLC22A7* expression, leading to a reduction in the transportation of orotic acid [41]. Adult male alcoholics are found to have elevated urinary orotic acid levels that decline with time following abstinence [43]. An experimental study provided evidence that alcoholism and various other diseases alter hepatocellular excretion of orotic acids, which can promote liver carcinogenesis after partial hepatectomy [44]. Further research is needed to fully elucidate the mechanisms that underlie liver carcinogenesis.

In conclusion, HCC was found to recur less frequently in the cases of NBNC HCC than in the cases of HCC with viral infection. Moreover, preoperative severe ALD was strongly associated with HCC recurrence after hepatectomy in NBNC patients.

Conflict of interest The authors declare that they have no conflict of interest.

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Clinical Science

Age-related clinicopathologic and molecular features of patients receiving curative hepatectomy for hepatocellular carcinoma

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KEYWORDS:
Hepatocellular carcinoma;
Aged;
Gene expression;
Fibrosis;
Pre-existing comorbidity;
Non-B non-C hepatocellular carcinoma

Abstract

BACKGROUND: Age-related differences of clinicopathologic features, outcomes, and molecular properties of hepatocellular carcinoma remain unclarified.

METHODS: We classified patients who underwent hepatectomy for hepatocellular carcinoma into 3 groups by age bracket; younger group (<50 years), middle-aged group (50 to 79 years), and elderly group (≥80 years) and compared age-related features.

RESULTS: Hepatitis viral infection was dominant in the younger group (hepatitis B virus [HBV]; 67%) and middle-aged group (hepatitis C virus [HCV]; 56%), whereas the elderly group showed a significantly higher rate without hepatitis virus infection (absence of HBV and HCV infection, 66%; $P = .0001$). There was a significantly greater proportion of age-associated pre-existing comorbidity in the elderly group (89%; $P = .0004$). Liver cirrhosis in the elderly group (24%) was significantly lower than other groups (younger, 67%; middle-aged, 50%; $P = .0058$). There was no significant difference in perioperative and postoperative outcomes among these groups. Microarray analysis revealed age-related upregulation of androgen and phosphatidylinositol 3-kinase pathways in the tumor tissue and downregulation of the fibrosis-related pathways in the noncancerous liver tissue.

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CONCLUSIONS: Based on increased correlation with the absence of HBV and HCV infection and pre-existing comorbidity, the age-related carcinogenic pathways might play a critical role in elderly hepatocarcinogenesis.

Hepatocellular carcinoma (HCC) is the fifth most common malignancy and one of the most common causes of cancer-related deaths in the world.[1,2] Recently, as the population is aging, the number of elderly HCC patients is increasing in the developed countries including Japan.[3] Although surgical resection is considered one of the main curative treatments of HCC,[4,5] the perioperative and postoperative outcomes in the elderly HCC patients have been controversial.[6–11] As the previous study suggested some different steps or mechanisms of hepatocarcinogenesis according to the patient's age-distribution,[12] the difference of pathologic features and molecular properties should be clarified.

In this study, we classified the patients who underwent hepatectomy for HCC into the 3 groups by age bracket, and compared the clinicopathologic features among the groups. Then, the perioperative morbidities were evaluated by Clavien-Dindo grading system, and postoperative outcomes were evaluated recurrence-free and overall survivals. Additionally, the genome-wide gene expression correlated with aging was analyzed by deoxyribonucleic acid (DNA) microarray that offers a systematic approach to acquire comprehensive information regarding gene transcription profiles.[13] Our study identified a strong correlation of aging with non-viral status and specific gene expression in the elderly HCC.

Methods

Patients and samples

We enrolled 486 patients who underwent curative hepatectomy for HCC at the Tokyo Medical and Dental University Hospital between April 2000 and February 2012. Written informed consent was obtained from these patients, and the institutional review board approved this study (#1080). The patients were classified into 3 groups by age bracket at the time of operation: younger group (<50 years), middle-aged group (50 to 79 years), and elderly group (≥80 years). We compared background characteristics, liver function data, tumor factors, perioperative outcomes, disease-free survival, and overall survival among these groups. With respect to personal lifestyles and societal conditions associated with economic development in elderly patients, we defined diabetes mellitus, hypertension, and dyslipidemia as an age-associated pre-existing comorbidity.[14]

DNA microarray analysis

The tissue preparation was essentially compliant with the General Rules for the Clinical and Pathological Study of Primary Liver Cancer. Total ribonucleic acid (RNA) was extracted from the HCC specimens with RNeasy kit (Qiagen, Hilden, Germany). The integrity of the RNA obtained was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). In this study, 178 tumor and 118 noncancerous liver tissues were available for analysis of gene expression. Contaminating DNA was removed by digestion with RNase-free DNase (Qiagen), and with 2 μg of total RNA, complementary RNA was prepared with a 1-cycle target labeling and control reagents kit (Affymetrix, Santa Clara, CA). The hybridization and signal detection of the Human Genome U133 (HG-U133) Plus 2.0 arrays (Affymetrix) were performed in accordance with the manufacturer's instructions. A total of 296 microarray data sets were normalized by the robust multiarray average method (R statistical software version 2.12.1 and the BioConductor package), essentially as described in our previous report.[15] The estimated gene expression levels were log 2 transformed, and a Pearson correlation test was performed to estimate the significance levels of the association between gene expression pattern and age. To remove genes with low variance across the samples, interquartile range (IQR) was calculated for each probe set. In tumor tissue, genes sets satisfying both $P < .001$ and IQR > 2 and in noncancerous tissue gene sets satisfying both $P < .01$ and IQR > 1.5 were considered as significant. The gene expression changes of 78 probe sets in the tumor tissue and 27 probe sets in the noncancerous tissue were evaluated in order of the P values and IQR.

Statistical analysis

Statistical comparisons of the clinicopathologic characteristics for significance were performed by the chi-square test or the Fisher exact test with a single degree of freedom, and a Student t test was used to analyze the differences between continuous values. Overall survival and disease-free survival were determined by the Kaplan-Meier method, and for comparisons, log-rank tests were used. P values less than .05 were considered to have statistical significance.

Results

Classification of patients with hepatocellular carcinoma

In this study, 486 patients who underwent curative hepatectomy for HCC were classified into 3 groups by age bracket: 24 cases in the younger group (<50 years), 433 cases in the middle-aged group (50-79 years), and the remaining 29 cases in the elderly group (≥80 years). The

Table 1 Clinical features

Characteristic	<50, *n* = 24	50–79, *n* = 433	≥80, *n* = 29	*P* value
Sex, *n* (%)				
Male	18 (75)	333 (77)	19 (66)	.3758
Female	6 (25)	100 (23)	10 (34)	
Hepatitis virus, *n* (%)				
HBsAg positive	16 (67)	73 (17)	1 (3)	<.0001*
HCVAb positive	3 (13)	241 (56)	9 (31)	<.0001*
NBNC	5 (21)	123 (28)	19 (66)	<.0002*
Age-associated pre-existing comorbidity	7 (33)	244 (60)	24 (89)	.0004*
Diabetes mellitus	3 (14)	115 (28)	10 (37)	.2171
Hypertension	3 (14)	183 (45)	19 (70)	.0003*
Dyslipidemia	2 (10)	60 (15)	4 (15)	.8060
Alcohol addicts	8 (38)	169 (41)	6 (22)	.1435
BMI >25	7 (33)	122 (30)	7 (26)	.8441

BMI = body mass index; HBsAg = hepatitis B antigen; HCVAb = hepatitis C antibody; NBNC = absence of hepatitis B virus and hepatitis C virus infection.
*P < .05.

mean age of the 3 groups was 43.5 ± 6.3 years (ranged from 24 to 49 years), 67.4 ± 7.1 years (ranged from 50 to 79 years), and 81.7 ± 1.7 years (ranged from 80 to 85 years), respectively. The mean follow-up period for the 3 groups was 1173 ± 818 days (ranged from 58 to 3175 days), 1195 ± 994 days (ranged from 1 to 4306 days), and 575 ± 622 days (ranged from 19 to 2771 days), respectively. The proportion of the elderly group in the late period (2007 to 2012) was significantly higher than that in the early period (2000 to 2006; 2.4% vs 8.5%; $P = .0160$).

The background characteristics of the enrolled patients are summarized in Table 1. There was a significantly higher

Table 2 Liver function and tumor factors

Characteristic	<50, *n* = 24	50–79, *n* = 433	≥80, *n* = 29	*P* value
Liver function, *n* (%)				
AST >50	5 (21)	173 (40)	11 (39)	.1610
ALT >50	6 (25)	154 (36)	7 (24)	.2596
PT% >70	22 (92)	406 (94)	25 (86)	.2480
Alb <3.5	2 (8)	86 (20)	3 (10)	.3409
T-bil >1.5	2 (8)	33 (8)	4 (14)	.4951
Liver damage A	20 (83)	314 (73)	23 (79)	.3855
Child-Pugh A	23 (96)	392 (91)	24 (83)	.2522
ICG-R_{15} ≥20	4 (17)	167 (39)	7 (24)	.0329*
Liver cirrhosis	16 (67)	216 (50)	7 (24)	.0088*
AFP ≥200	12 (50)	97 (22)	6 (21)	.0122*
PIVKA-II ≥200	11 (46)	164 (38)	17 (59)	.0762
Tumor size ≥5 cm	12 (50)	153 (35)	15 (52)	.0842
Number of tumor ≥2	10 (33)	156 (35)	12 (40)	.8211
Vascular or biliary invasion positive	17 (71)	208 (48)	12 (43)	.0458*
pva ≥1	0 (0)	3 (1)	1 (3)	.1203
pvp ≥2	10 (42)	50 (12)	4 (14)	.0001*
pvv ≥2	1 (4)	11 (3)	2 (7)	.3515
pb ≥2	1 (4)	10 (2)	1 (4)	.7884
Differentiation, *n* (%)				
moderate + poorly	21 (91)	299 (72)	15 (54)	.0119*
CLIP score ≥2	12 (50)	119 (28)	10 (36)	.0456*
JIS score ≥2	21 (72)	251 (56)	21 (70)	.0779
Stage, *n* (%)				
III + IV	17 (71)	240 (55)	17 (59)	.3234

AFP = alpha-fetoprotein; Alb = albumin; ALT = alanine aminotransferase; AST = aspartate aminotransferase; CLIP = Cancer of the Liver Italian Program; ICG-R_{15} = indocyanine green retention rate at 15 minutes; JIS = Japan Integrated Staging; PIVKA-II = protein induced by vitamin K absence or antagonist II; PB = biliary invasion; PT = prothrombin time; pva = pathologic findings of arterial invasion; pvp = portal vein invasion; pvv = hepatic vein invasion; T-bil = total bilirubin.
*P < .05.

Table 3 Perioperative outcome

Characteristic	<50, n = 24	50–79, n = 433	≥80, n = 29	P value
Operative procedure, n (%)				
Anatomic resection	18 (75)	297 (69)	24 (83)	.2328
Nonanatomic resection	6 (25)	136 (31)	5 (17)	
Length of stay, >30 days	1 (4)	73 (17)	4 (14)	.2423
Morbidity, n (%)				
Overall morbidity	8 (33)	165 (38)	15 (52)	.3028
Clavien-Dindo morbidity IV or V	0 (0)	36 (8)	3 (10)	.3075

positive rate of hepatitis B antigen in the younger group, and that of hepatitis C antibody in the middle-aged group, whereas the absence of hepatitis B virus (HBV) and hepatitis C virus (HCV) infection (NBNC) was significantly higher in the elderly group. Additionally, there was a significantly greater proportion of pre-existing comorbidity in the elderly group (89%) compared with the other groups (P = .0004). The alcohol addicts were lower in proportion in the elderly group than in the middle-aged group (P = .0423). There was no significant difference in sex and the proportion of participants with body mass index > 25 among these groups.

Liver functions and tumor factors

The data of liver functions are summarized in Table 2. Indocyanine green retention rate at 15 minutes (ICG-R_{15}) in the younger group was significantly lower than middle-aged group (P = .0329). Liver cirrhosis proven by histologic diagnosis in the elderly group (24%) was significantly lower than other groups (P = .0058). There was no significant difference between these groups in the proportion of participants with respect to the aspartate aminotransferase level, alanine aminotransferase level, prothrombin time, albumin level, total bilirubin level, liver damage, or Child-Pugh classification.

The data of tumor factors are also summarized in Table 2. Serum alpha-fetoprotein (AFP) level in the younger group was significantly higher than in other groups (P = .0122). The rate of portal vein invasion in the younger group (42%) was significantly higher than in other groups (P = .0001). The rate of moderately or poorly differentiated HCC in the younger group was significantly higher than in other groups (P = .0119). The Cancer of the Liver Italian Program system[16,17] showed a significantly higher score in the younger group than in other groups (P = .0456). It was attributed to higher serum AFP level and presence of vascular invasion rate. There was no significant difference among these groups in the proportion of participants with the serum protein induced by vitamin K absence or antagonist II level, tumor size, number, the Japan Integrated Staging score,[18] or TNM Stage.

Perioperative and postoperative outcomes

Perioperative outcomes are summarized in Table 3. There was no significant difference in operative procedures. Anatomic resection was performed for 24 of 29 cases (83%) in the elderly group. The length of stay after operation of each group was 16.6 ± 10.4 days (younger), 22.0 ± 26.9 days (middle-aged), and 20.4 ± 19.2 days (elderly), indicating no significant difference. No significant difference was observed in either overall morbidity or Clavien-Dindo grade-IV or -V morbidity[19] among these 3 groups. In the elderly group, 15 patients (52%) experienced any complications. The most common complication was delirium (24%), followed by bile leakage (14%). The length of hospital stay after surgery was 29.1 days (ranged from 5 to 95 days) in the presence of complications, compared with 11.1 days (ranged from 7 to 16 days) in the absence of complication. There was no significant difference in disease-free survivals (Fig. 1A) as well as in overall survivals (Fig. 1B) among these 3 groups. The 5-year survival rate was 60% (younger), 58% (middle-aged), and 46% (elderly), respectively. Overall survivals according to TNM Stage were also not significantly different (Supplementary Fig. 1A–C).

Genome-wide gene expression analysis correlated to aging

Age-related gene expression was analyzed in the main tumor of HCC and noncancerous liver tissue samples. As shown in Fig. 2A,C, the alteration of gene expression demonstrated clearly in accordance with the aging in the tumor as well as liver tissue. In tumor tissue (Fig. 2B), the network with androgen receptor (AR) and phosphatidylinositol 3-kinase regulatory subunit 1 (PI3KR1) was significantly upregulated with aging. In liver tissue (Fig. 2D), the network with mothers against decapentaplegic Drosophila homolog 3 (SMAD3), SMAD4, cAMP response element binding (CREB) protein, and fibroblast growth factor receptor 1 (FGFR1) were significantly downregulated with aging.

Comment

Patients with HCC are known to consist of heterogeneous populations.[2] In the present study, the patients who underwent surgical operation for primary HCC were classified into the younger, middle-aged, and elderly groups. There have been several reports regarding the difference of perioperative and postoperative outcomes in HCC patients in the

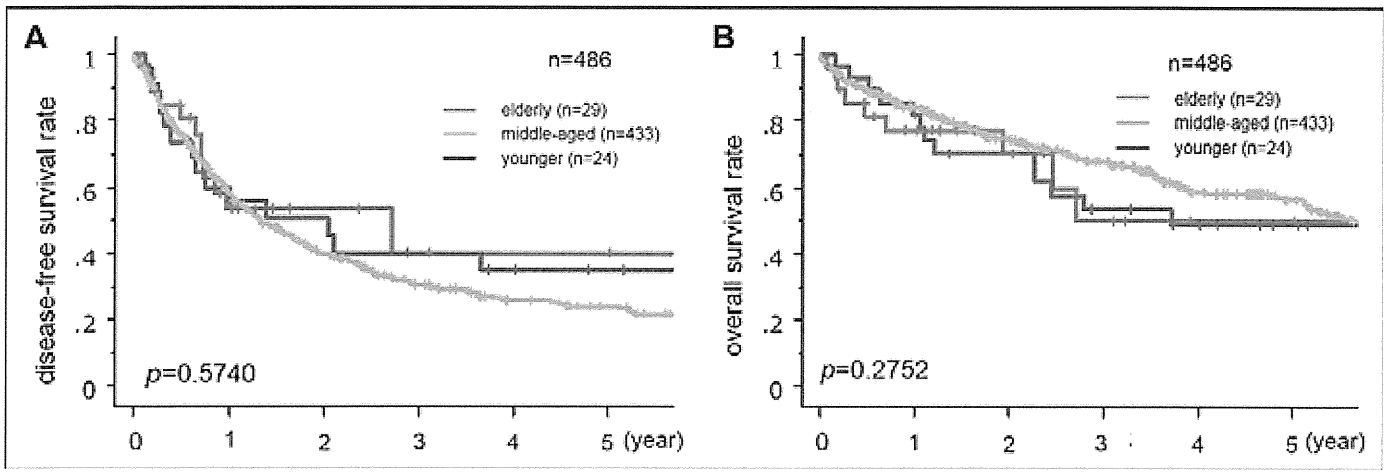

Figure 1 (A) Disease-free survival and (B) overall survival of patients with HCC according to the age.

elderly.[6–11] Our study revealed that there were no significant differences in the outcomes in the elderly group compared with the middle-aged and younger group, although some selection bias should be considered for our surgical indication in the elderly patients (Fig. 1). Morbidity and mortality were not correlated with the operative risks using the Physiologic and Operative Severity Score for the enUmeration of Mortality and Morbidity (POSSUM) scoring system[20] (data not shown). Although clinical outcomes were not different, several clinicopathologic features were varied among the 3 groups. The analysis of background liver diseases characterized the younger group as HBV type, the middle-aged group as HCV type, and the elderly group as NBNC type (Table 1). Previous studies demonstrated that the average age of diagnosis of HBV-related HCC was lesser than that of HCV-related HCC.[21,22] In other reports, patients with NBNC HCC were significantly older than HCC occurring in a background of viral hepatitis B and C.[12,23]

The HCC patients in the younger group showed higher rate of AFP elevation, tumor with vascular invasion, and moderate to poor differentiation (Table 2). According to the previous studies, HBV HCC frequently showed with high AFP levels or vascular invasion[12,24–26] that were associated with the histologic poor differentiation of HCC.[6] As shown

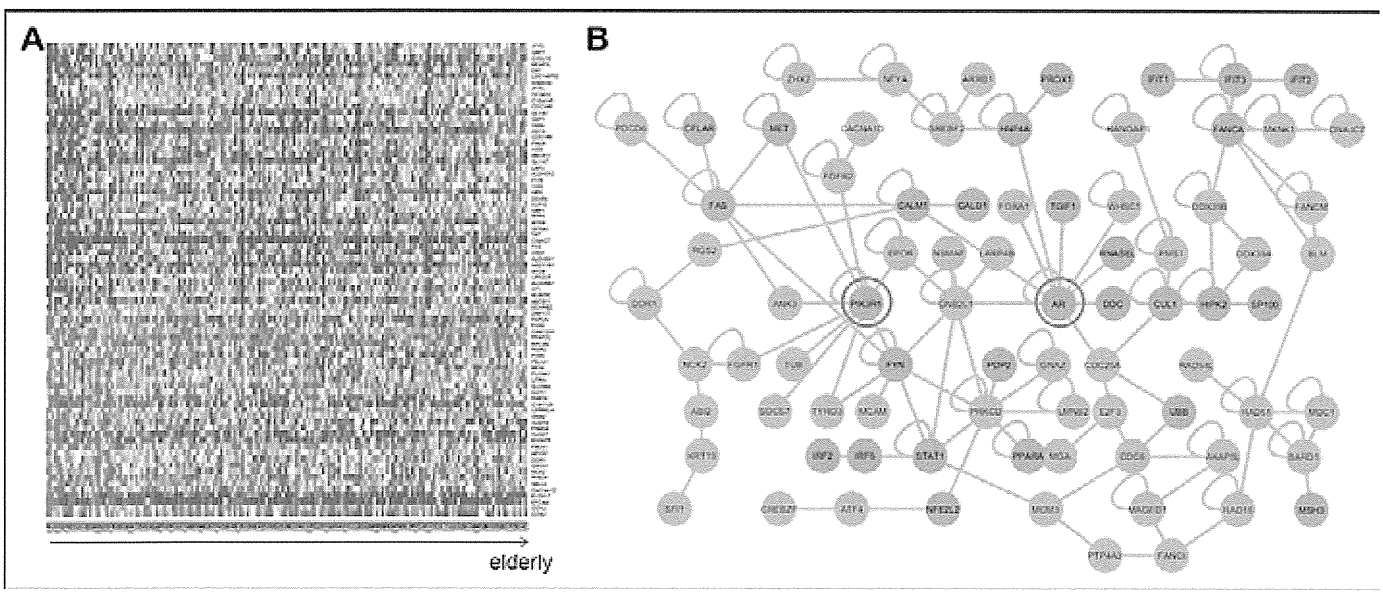

Figure 2 (A) Molecular and biological analysis in relation to the age of HCC in tumor tissue. Expression profiling of genes correlated with age. The hierarchical clustering of 78 age-associated genes ($P < .001$ and IQR > 2) in tumor tissue was performed. The red and blue areas indicate relative overexpression and underexpression, respectively. (B) A protein interaction network constructed using 999 probe sets correlated with age ($P < .01$ and IQR > 1.5); the largest sub-network containing 80 genes was extracted. (C) Molecular and biological analysis in relation to the age of HCC in noncancerous tissue. Expression profiling of genes correlated with age. The hierarchical clustering of 27 age-associated genes ($P < .01$ and IQR > 1.5) in non-cancerous tissue was performed. The red and blue areas indicate relative overexpression and underexpression, respectively. (D) A protein interaction network constructed using 1,141 probe sets correlated with age ($P < .01$); the largest sub-network containing 117 genes was extracted. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

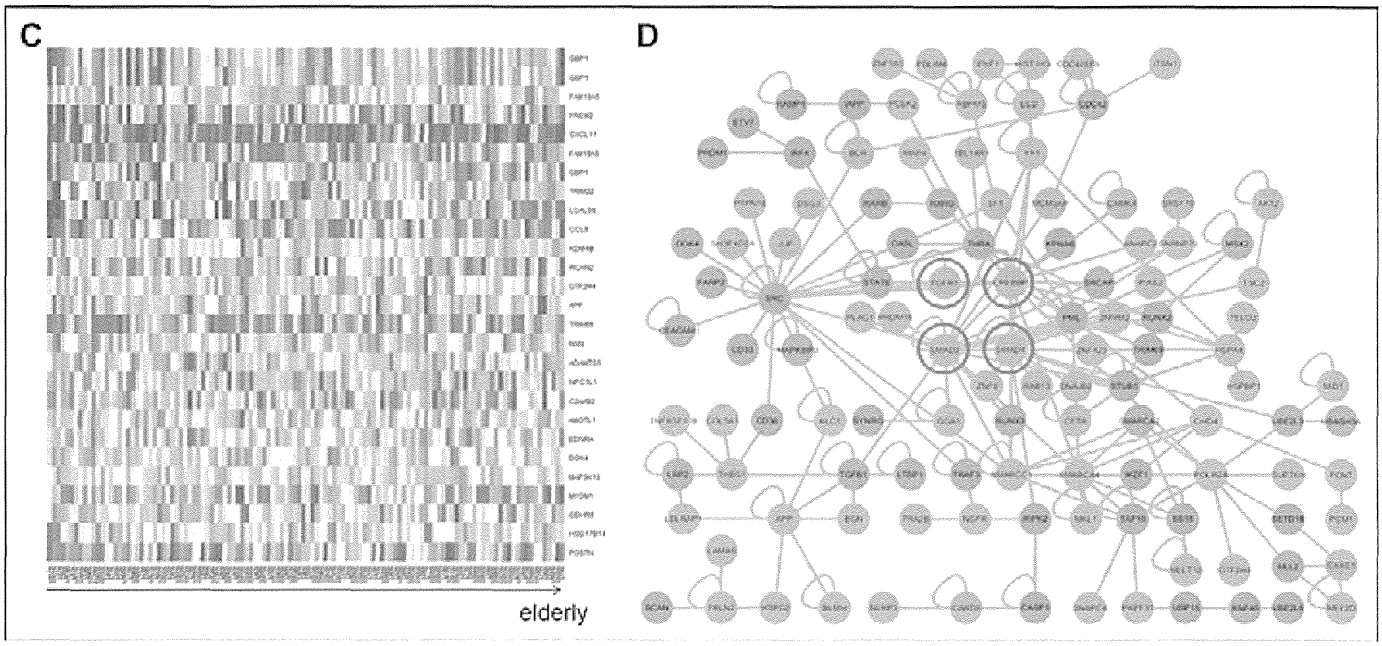

Figure 2 *(Continued)*

in Table 2, the middle-aged group showed higher ICG-R_{15} levels and cirrhotic rates. It is compatible with the previous reports that HCC patients with hepatitis C antibody frequently suffered from liver dysfunction compared with those with other types of HCC.[24] Interestingly, the elderly group was not related to viral hepatitis or severe cirrhosis but closely to the pre-existing comorbidity (Table 1). Our results were compatible with the previous report indicating that the mechanisms of hepatocarcinogenesis may be significantly different in accordance with aging.[12,27]

To clarify the molecular and biological features of age-related hepatocarcinogenesis, the gene expression profiling was further evaluated in HCC samples. The gene expression analysis identified AR and PI3KR1 as dominant pathways upregulated in the tumor tissues with the aging (Fig. 2A,B). The overexpression of AR was strongly associated with intrahepatic recurrence of HCC,[28] and the specific knockout of AR significantly reduced tumorigenicity of HCC in the mouse models.[27] PI3KR1, a regulatory subunit of PI3K,[29] is involved in the oncogenic signaling of PI3K/Akt/mammallian Target Of Rapamycin (mTOR).[30] We have previously identified the critical role of PI3K pathway in cancer proliferation and survival[31] in human HCC.[32] Zheng et al[33] reported that PI3KR1 was frequently overexpressed in HCC tissues than adjacent noncancerous tissues, and the PI3KR1 knockdown–induced cell apoptosis in human HCC. Recently, the clinical phase I study of mTOR inhibitor everolimus revealed its potential effectiveness to treat patients with advanced HCC.[34] On the other hand, the gene expression analysis of noncancerous liver tissue revealed that fibrosis-related pathways were downregulated with aging; including the SMAD3 and 4 signaling factors and CREB transcriptional coactivator of transforming growth factor beta, as well as the FGFR1 (Fig. 2C,D). In the elderly NBNC patients, the specific oncogenic pathways might play a role in hepatocarcinogenesis, although the microenvironment was different from the other patients with viral hepatitis and liver fibrosis.

Conclusions

Our study identified the age-related differences of clinicopathologic and molecular properties in HCC patients. It might suggest that there are different mechanisms of hepatocarcinogenesis, indicating theoretically therapeutic strategy for HCC by age. Potential combination of antiandrogen and PI3K/Akt/mTOR pathway–targeted therapies could be effective and it would be worth considering as a molecular target for specific adjuvant therapy in elderly patients with HCC.[35] Further studies should be focused on the oncogenic mechanisms and therapeutic targets in the elderly HCC.

Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.amjsurg.2014.01.015.

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ORIGINAL ARTICLE – HEPATOBILIARY TUMORS

EpCAM-Targeted Therapy for Human Hepatocellular Carcinoma

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ABSTRACT

Background. Hepatocellular carcinoma (HCC) is one of the most lethal malignancies and the identification of new effective therapies for HCC is urgently needed. We have previously identified EpCAM, one of the hepatic stem/progenitor markers, as a prognostic predictor of patients who received curative hepatectomy for HCC. In this preclinical study, the effects of VB4-845, an immunotoxin targeting EpCAM, were evaluated in HCC.

Methods. In vitro effects of VB4-845 on human HCC cells, the cytotoxic activity, sphere-forming ability, and expression of hepatic stem/progenitor markers were analyzed. In vivo effects of VB4-845 were evaluated using subcutaneous and orthotopic liver xenograft models.

Results. In all HCC cell lines expressing EpCAM, VB4-845 showed potent cytotoxicity and was significantly effective in combination with 5-FU ($p < 0.05$). Although 5-FU did not affect the sphere-forming ability and increased the populations expressing other stem/progenitor markers CD133 and CD13 ($p < 0.05$), VB4-845 strongly suppressed the sphere-formation and decreased the population expressing CD133 and CD13 ($p < 0.0005$, <0.01, respectively). In subcutaneous xenograft models, the combination of VB4-845 plus 5-FU showed significant regression of tumors compared with the control ($p = 0.016$). Moreover, in orthotopic liver xenograft models, the combination therapy dramatically decreased the tumor volume compared with the control ($p = 0.0011$).

Conclusions. Our preclinical investigation suggests that EpCAM-targeted therapy may offer a promising and novel approach for the treatment of HCC with a poorer prognosis.

Hepatocellular carcinoma (HCC) is the fifth most common cancer and one of the leading causes of cancer death worldwide.[1] Although the primary curative treatment for HCC is surgical resection, including liver transplantation, various therapeutic options have been employed, including radiofrequency ablation, transarterial chemoembolization, and chemotherapy (5-FU).[2,3] Effective palliative treatment is hindered by the fact that HCC is frequently resistant to conventional cytotoxic agents. Sorafenib has demonstrated improved overall survival in patients with advanced HCC.[4] However, the median overall survival among patients with advanced HCC is still less than 1 year and the prognosis remains poor.[5]

A recent report revealed that malignant tumors with poor prognosis showed preferential overexpression of genes normally enriched in embryonic stem cells using expression analyses of gene sets.[6] Indeed, several studies revealed that hepatic stem/progenitor markers, including EpCAM, CD133, CD44, and CD90 were the biomarkers of HCC with poor prognosis.[7–10] So, cancer cells expressing stem/progenitor markers might be recognized as the critical targets for the treatment of HCC. On the other hand, EpCAM, CD133, CD13, CD44, and CD90, previously identified as hepatic stem/progenitor markers, have been used for isolation of cancer stem cells that should carry indefinite potential for self-renewal that drive tumorigenesis.[11–16]

We have previously reported that EpCAM might be a biomarker of HCC with confluent multinodular (CM) type that has been identified morphologically as a poor

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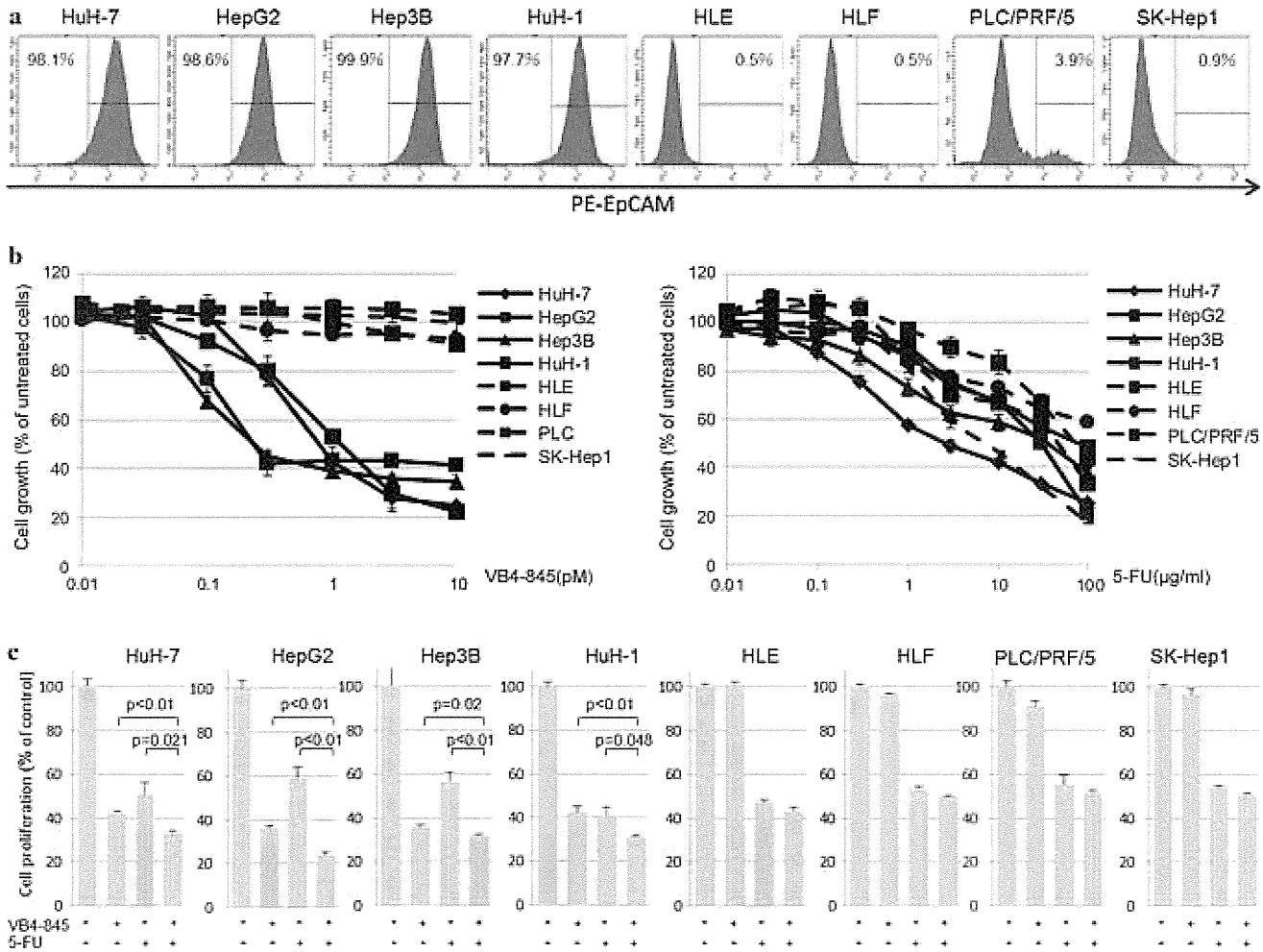

FIG. 1 Association of EpCAM expression with in vitro effects of VB4-845 and 5-FU in human HCC cells. **a** Expression of EpCAM was analyzed by flow cytometry in 8 HCC cell lines and the positive rate of EpCAM was indicated. **b** Inhibition of tumor cell growth upon treatment with VB4-845 or (**c**) 5-FU. *Error bars*, deviation. **d** Cell proliferation assay of eight HCC cell lines with or without VB4-845 and 5-FU for 48 h. *Columns*, alive cells (%); *vertical bars*, standard deviation. All experiments were performed in triplicate

prognostic factor of HCC.[17–19] In this study, we focused on the expression of EpCAM in human HCC cell lines and analyzed the effects of VB4-845 (Oportuzumab monatox), an immunotoxin targeting EpCAM, on human HCC cell lines. VB4-845 is a recombinant fusion protein comprising a humanized anti-EpCAM single-chain antibody linked to Pseudomonas exotoxin A.[20] Several clinical trials employing VB4-845 have been conducted in patients with head and neck squamous cell carcinoma and bladder cancer.[21,22] However, the effects of VB4-845 on HCC cells have not been determined. In this report, we demonstrated that EpCAM was highly expressed in several HCC cells and that VB4-845 suppressed their sphere-forming ability and the positive rates of stem/progenitor markers. VB4-845 dramatically suppressed the tumor growth under the combination of 5-FU on subcutaneous and orthotopic liver xenograft models.

MATERIALS AND METHODS

Cell Culture and Flow Cytometry

Human HCC cell lines HuH-1, HuH-7, HepG2, Hep3B, HLE, HLF, PLC/PRF/5, and SK-Hep1 were prepared as described previously.[23] Luciferase expression plasmid pGL4.50 [luc2/CMV/Hygro] (#E131A; Promega, Madison, WI) was transfected into HuH-7 cells according to the manufacturer's instructions and luciferase-expressing HuH-7 cells (HuH-7-*Luc*) were generated. For flow cytometry, FACSCanto^{TM}II (BD Biosciences, San Jose, CA, USA) was used as described previously.[23,24] For the analysis of hepatic stem/progenitor markers, primary antibodies against EpCAM (#324206; Biolegend, San Diego, CA), CD13 (#555394; BD Pharmingen), CD44 (#555479; BD Pharmingen), CD90 (#328110; BioLegend), CD133

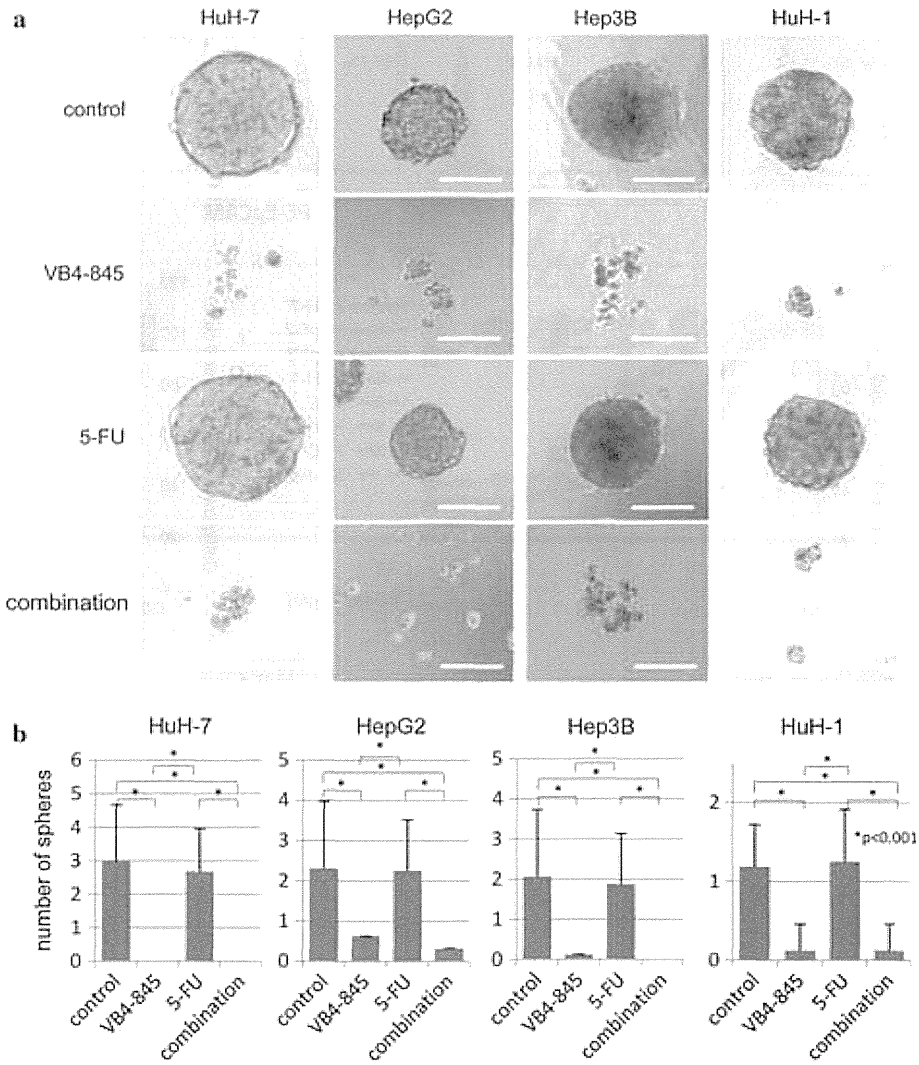

FIG. 2 Sphere formation in EpCAM^{high} cell lines after the treatment of VB4-845 (1 pM), 5-FU (5 μg/ml), and the combination of VB4-845 plus 5-FU using 3D culture system. **a** Representative microscopic image of spheres (×100). *Scale bar*, 100 μm. **b** The number of sphere (>100 μm in diameter) observed in each well after drug administration ($n = 16$ in each). Control cells and the surviving cells after the treatment of 5-FU formed spheres, whereas the surviving cells after the treatment of VB4-845 or combination did not form spheres. *Columns*, average number of sphere; *vertical bars*, standard deviation

(#130-080-801; Miltenyi Biotec, Gladbach, Germany), Mouse IgG1 κtype (#555749; BioLegend), and Mouse IgG2b κtype (#400314; BioLegend) were used.

Analysis of Cell Proliferation and Viability

VB4-845 was provided by Viventia Bio Inc. (Winnipeg, Manitoba Canada). HCC cell lines were seeded in 96-well plates at 3×10^3 cells per well. After 24 h, VB4-845 concentrations ranging from 0.001 to 10 pM were added and incubated for 72 h, or 5-FU concentrations ranging from 0.01 to 100 μg/ml were added and incubated for 48 h. Using Cell Titer 96 AQueous One Solution Cell Proliferation Assay Kit (Promega), half-maximal inhibitory concentration (IC_{50}) values were calculated in triplicate as described in our previous reports.[23] To investigate cell viability, HCC cells were seeded in 6-well plates at 1×10^5 cells per well. After 24 h, VB4-845 (1 pM) and/or 5-FU (5 μg/ml) were added and incubated for 48 h. The remaining viable cells were counted by trypan blue exclusion using a Cytorecon (GE Healthcare).

Sphere Formation Assay

The sphere formation assay was performed as previously described.[24,25] Briefly, 1×10^6 cells of HuH-7, HepG2, Hep3B, and HuH-1 were seeded in four 10-cm dishes. After 24 h, PBS, VB4-845 (1 pM), 5-FU (5 μg/ml), and a combination of VB4-845 plus 5-FU were administered in each dish. After 48 h, the medium was changed to drug free medium and incubated for 24 h. Using trypan blue exclusion, the remaining viable cells were collected and plated separately at 1×10^2 cells in low attachment plates (96-well Ultra Low Cluster Plate; Costar, Corning, NY), and incubated in serum-free medium ($n = 16$ in each). Sphere formation was observed using AxioObserver (Carl Zeiss, Oberkochen, Germany), and the images were acquired digitally using AxioVision software (Carl Zeiss).