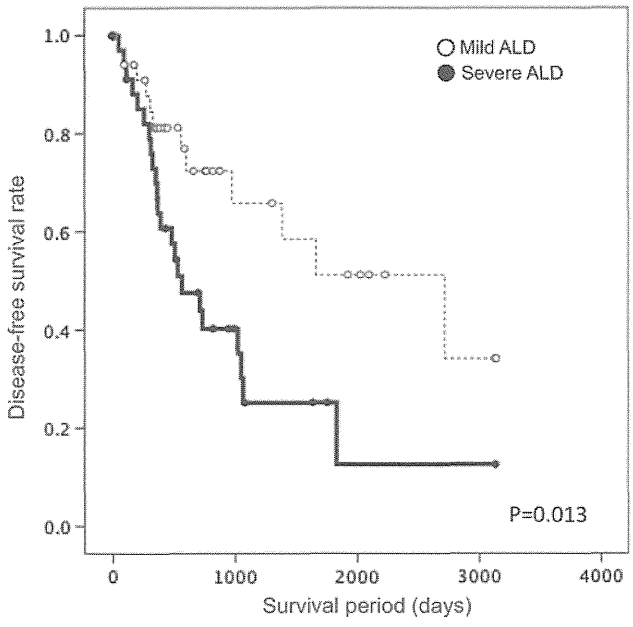

Fig. 2 Disease-free survival of non-B non-C patients in severe and mild alcohol liver disease (ALD) groups before adjustment with propensity scores. *Open* and *closed circles* denote the mild and severe ALD patients, respectively. The difference between the two groups was remarkable ($P = 0.013$)

($P = 0.034$), 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/μL)	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

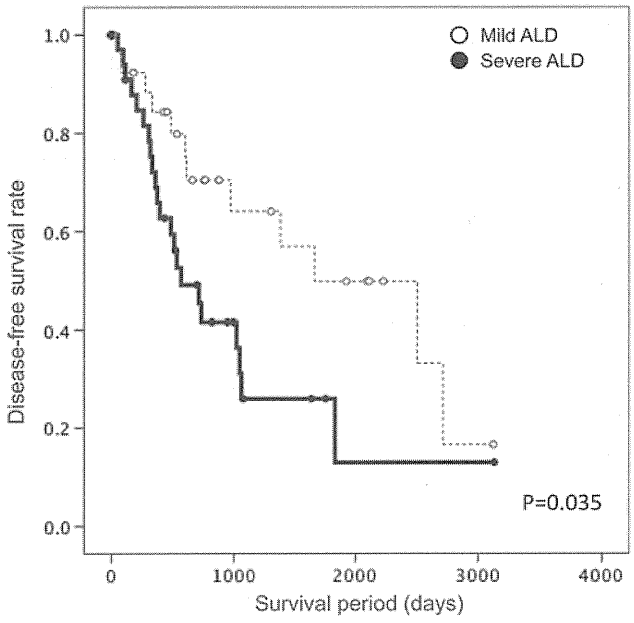

Fig. 3 Disease-free survival rates of non-B non-C patients in severe and mild alcohol liver disease (ALD) groups after adjustment with propensity scores. *Open* and *closed circles* denote the mild and severe ALD groups, respectively. The difference between the two groups was remarkable ($P = 0.035$)

(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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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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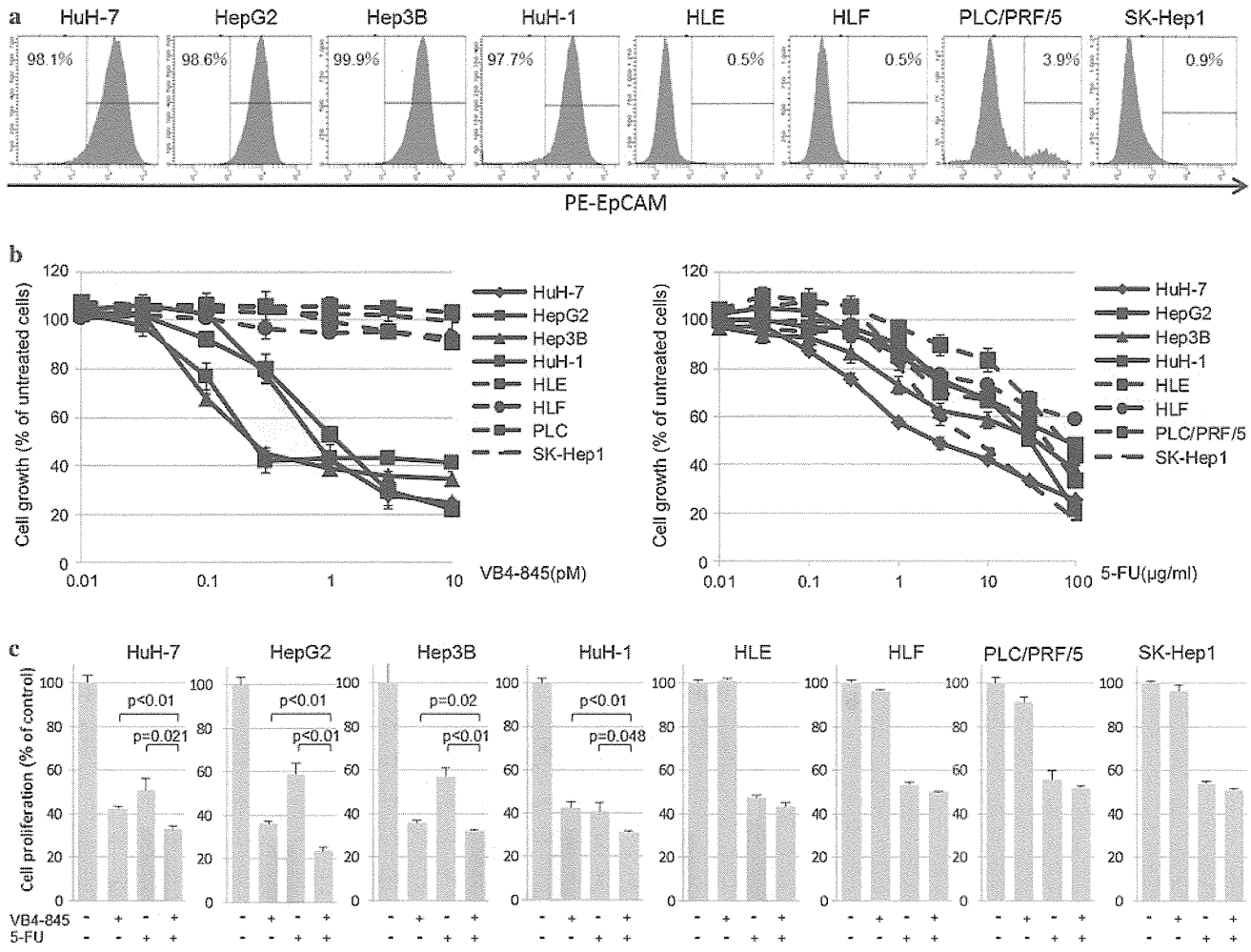


FIG. 1 Association of EpCAM expression with in vitro effects of VB4-845 and 5-FU in human HCC cells. **a** Expression of EpCAM was analyzed by flow cytometry in 8 HCC cell lines and the positive rate of EpCAM was indicated. **b** Inhibition of tumor cell growth upon treatment with VB4-845 or (**c**) 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

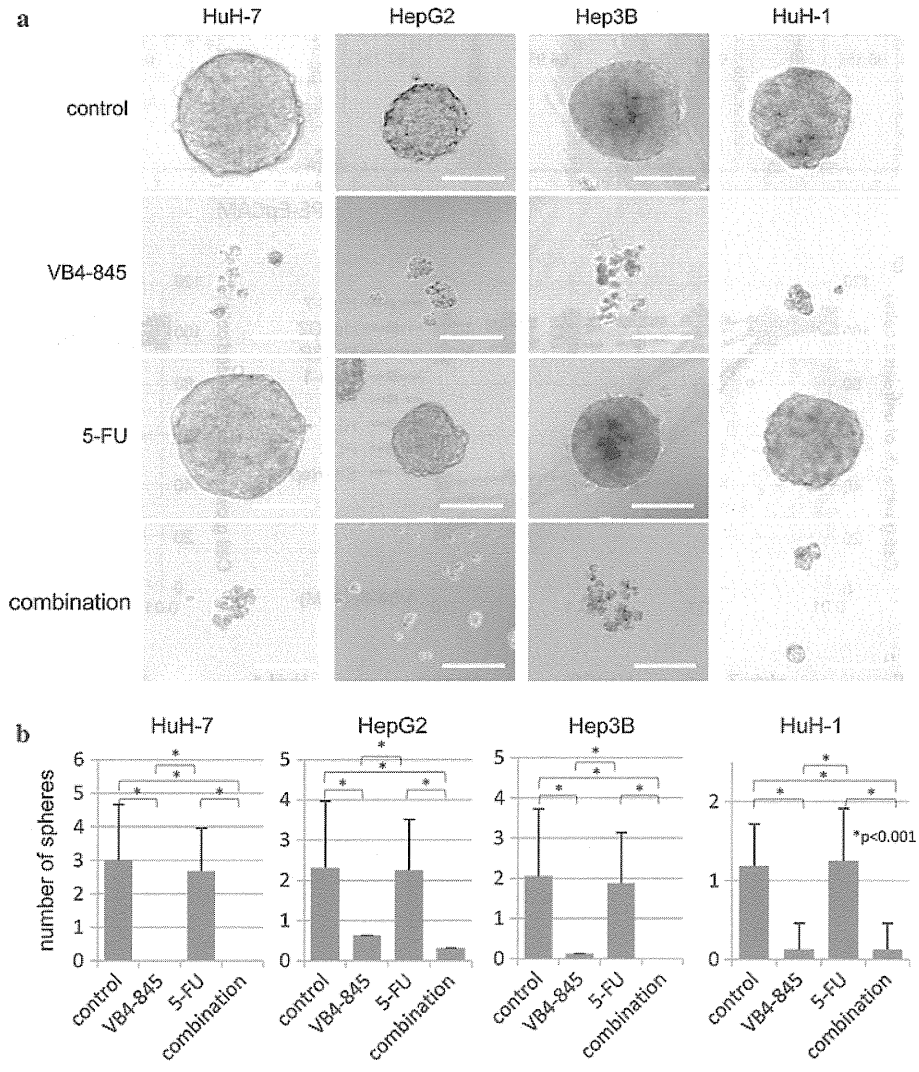


FIG. 2 Sphere formation in EpCAM^{high} cell lines after the treatment of VB4-845 (1 pM), 5-FU (5 μg/ml), and the combination of VB4-845 plus 5-FU using 3D culture system. **a** Representative microscopic image of spheres (×100). *Scale bar*, 100 μm. **b** The number of sphere (>100 μm in diameter) observed 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

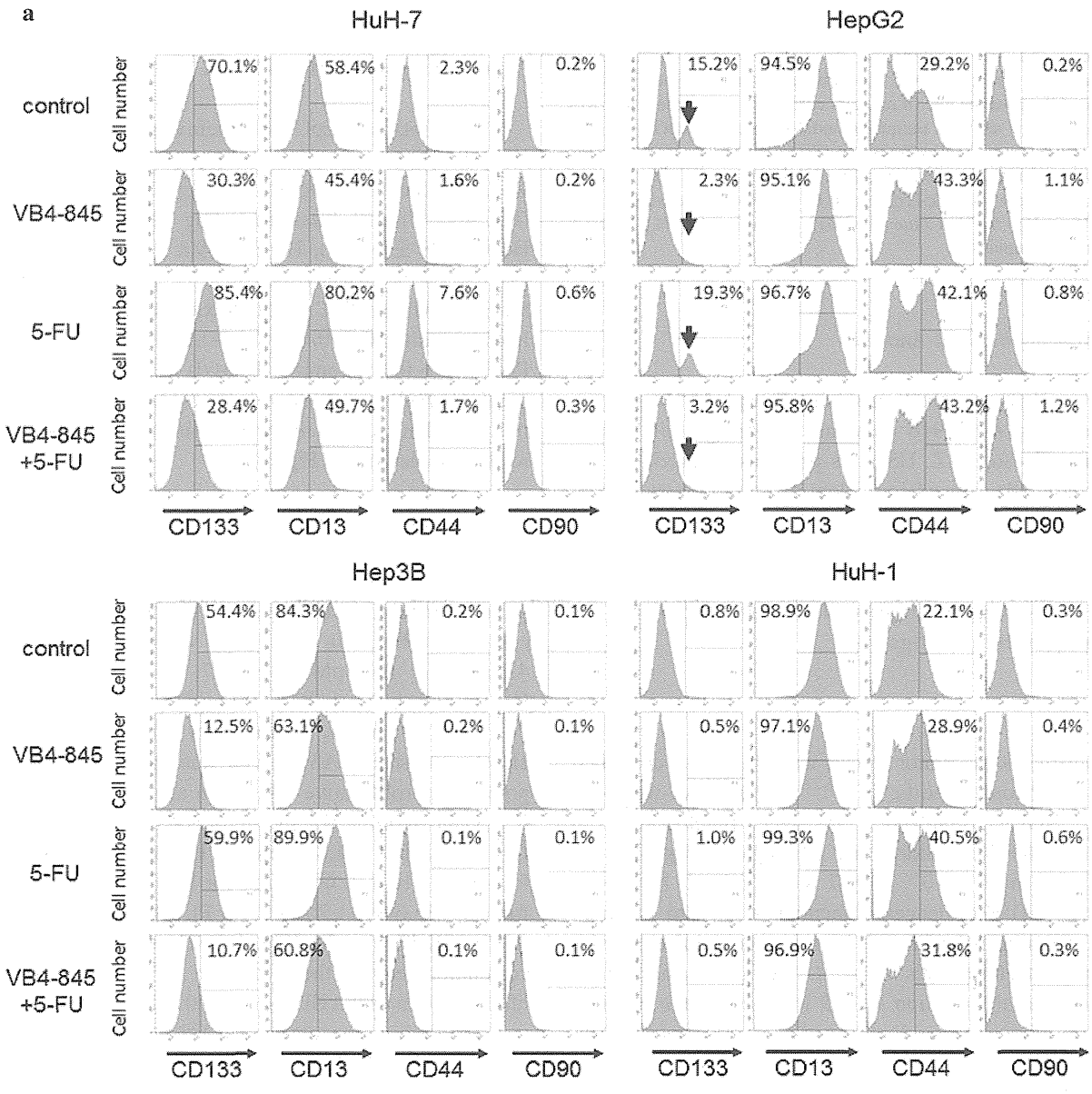
(#130-080-801; Miltenyi Biotec, Gladbach, Germany), Mouse IgG1 κtype (#555749; BioLegend), and Mouse IgG2b κtype (#400314; BioLegend) were used.

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).


a
HuH-7
HepG2
Hep3B
HuH-1
control
VB4-845
5-FU
VB4-845 +5-FU
Cell number
70.1%
58.4%
2.3%
0.2%
15.2%
94.5%
29.2%
0.2%
30.3%
45.4%
1.6%
0.2%
2.3%
95.1%
43.3%
1.1%
85.4%
80.2%
7.6%
0.6%
19.3%
96.7%
42.1%
0.8%
28.4%
49.7%
1.7%
0.3%
3.2%
95.8%
43.2%
1.2%
54.4%
84.3%
0.2%
0.1%
0.8%
98.9%
22.1%
0.3%
12.5%
63.1%
0.2%
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0.5%
97.1%
28.9%
0.4%
59.9%
89.9%
0.1%
0.1%
1.0%
99.3%
40.5%
0.6%
10.7%
60.8%
0.1%
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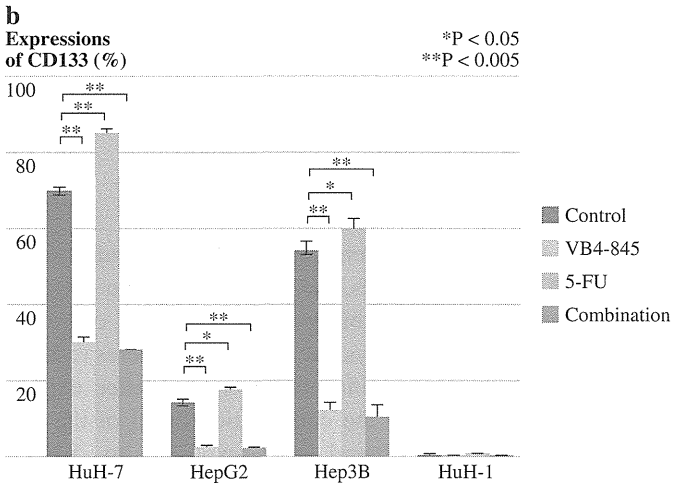
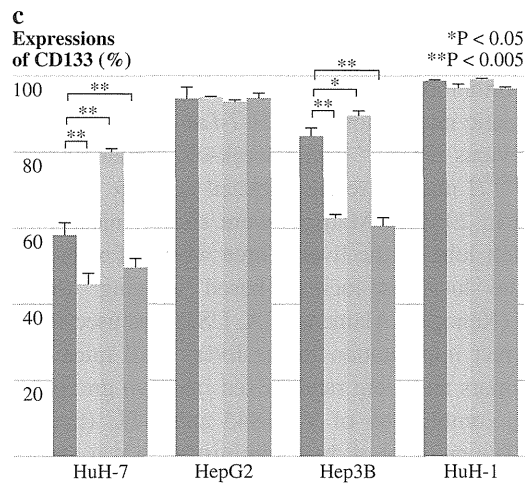
0.5%
96.9%
31.8%
0.3%
CD133
CD13
CD44
CD90


b
Expressions of CD133 (%)
*P < 0.05
**P < 0.005
100
80
60
40
20
HuH-7
HepG2
Hep3B
HuH-1
Control
VB4-845
5-FU
Combination


c
Expressions of CD133 (%)
*P < 0.05
**P < 0.005
100
80
60
40
20
HuH-7
HepG2
Hep3B
HuH-1

◀**FIG. 3** FACS analysis of EpCAM^{high} cell lines based on various stem/progenitor markers after the treatment of VB4-845 (1 pM), 5-FU (5 μg/ml), and the combination of VB4-845 plus 5-FU. **a** A representative result of three independent staining experiments is shown and the positive rate of markers corresponding to the graph was indicated. *Arrow* shows a unique bimodal pattern of HepG2 cells for CD133 expression. **b** Expression of CD133 after the treatment of VB4-845 and/or 5-FU. *Columns*, CD133 positive cells (%); *vertical bars*, standard deviation. **c** Expression of CD13 after the treatment of VB4-845 and/or 5-FU. *Columns*, CD13 positive cells (%); *vertical bars*, standard deviation

Immunohistochemical Analysis

Immunohistochemical analysis of EpCAM was performed using #ab71916 (Abcam, Cambridge, UK) using Ventana (Tucson, AZ, USA) as previously described.[17,26] The tumor cells showed equivalent membranous staining to normal bile duct epithelium was defined as strongly stained tumor cells. The immunostaining was evaluated quantitatively by counting in no fewer than three different random fields (100× magnification) under a light microscope by two independent investigators.

In Vivo Studies

A subcutaneous tumor model was created as described in our previous reports.[23,24,26,27] Twenty-five-week-old female NOD.CB17-PRkdc^{Scid}/J mice purchased from Charles River Laboratory Inc. (Kanagawa, Japan) were injected with 1×10^6 HuH-7 cells mixed half with Matrigel (BD Biosciences) subcutaneously into the both flanks under anesthesia and 16 mice were injected with 1×10^6 SK-Hep1 cells as well. Palpable tumors were confirmed in all injection sites 2 weeks after the inoculation, and mice were randomized into four groups: control, VB4-845 (30 μg/kg), 5-FU (30 mg/kg), and a combination of VB4-845 and 5-FU. VB4-845 diluted in 100 μl of saline was administered by tail vein injection, and/or 5-FU was injected intraperitoneally three times a week for 2 weeks.

An orthotopic xenograft model was created by direct intrahepatic inoculation of HuH-7-*Luc* cells, as described in our previous report.[23] Ten 5-week-old female NOD.CB17-PRkdc^{Scid}/J mice were anesthetized and 5×10^5 cells suspended in 20 μl of Matrigel were slowly injected into the upper left lobe of the liver. Three weeks after the inoculation, the luciferase-luciferin-based imaging using IVIS system (Xenogen, Alameda, CA, USa) was used to monitor the correct implantation in the liver.[28] All mice exhibited liver tumors and were randomized into two groups; control and the combination of VB4-845 and 5-FU (five mice in each). The protocol of drug administration was same as the subcutaneous model. All in vivo procedures were approved by the Animal Care Committee (permission #0130059A).

RESULTS

Expression of EpCAM in Human HCC Cells

The expression of EpCAM protein was assessed using FACS analysis in eight human HCC cell lines (Fig. 1a). These cell lines were divided into two groups: EpCAM high-expression (EpCAM^{high}) cell lines, including HuH-7 (98 ± 0.3 %), HepG2 (98 ± 0.9 %), Hep3B (99.8 ± 0.1 %), and HuH-1 (97.7 ± 0.2 %) in which more than 95 % of cells were positive for EpCAM; and EpCAM low-expression (EpCAM^{low}) cell lines, including HLE (0.4 ± 0.1 %), HLF (0.4 ± 0.2 %), PLC/PRF/5 (4 ± 0.3 %), and SK-Hep1 (0.7 ± 0.2 %) in which less than 5 % of cells were positive for EpCAM. The expression level of EpCAM in each cell line was confirmed immunocytochemically (Supplementary Fig. 1).

In Vitro Effects of VB4-845 and/or 5-FU

VB4-845, an immunotoxin targeting EpCAM,[20] was effective for EpCAM^{high} cell lines but not for the EpCAM^{low} cell lines (Fig. 1b). The IC_{50} value of VB4-845 was $4.6 \pm 0.1 \times 10^{-2}$ pM for HuH-7, $1 \pm 0.1 \times 10^{-2}$ pM for HepG2, $0.9 \pm 0.1 \times 10^{-2}$ pM for Hep3B, and $7.3 \pm 0.2 \times 10^{-2}$ pM for HuH-1. On the other hand, VB4-845 had no effect against EpCAM^{low} cell lines at all. 5-FU showed potent antiproliferative activity in all HCC cell types with IC_{50} value of 0.8 ± 0.1 μg/ml for HuH-7, 39.5 ± 9.6 μg/ml for HepG2, 5.9 ± 1.8 μg/ml for Hep3B, 11.3 ± 6.3 μg/ml for HuH-1, 16.5 ± 6.6 μg/ml for HLE, 33.5 ± 17.2 μg/ml for HLF, 55.6 ± 11.2 μg/ml for PLC/PRF/5, 4.3 ± 0.5 μg/ml for SK-Hep1 cells (Fig. 1c). There was no significant correlation between the efficacy of 5-FU and the expression of EpCAM in each cell line ($R = 0.16, p = 0.38$).

The combination effects of VB4-845 and 5-FU were assessed in eight human HCC cell lines (Fig. 1d). The combination of VB4-845 and 5-FU significantly suppressed cell proliferation in all of the EpCAM^{high} cell lines ($p < 0.05$). However, the EpCAM^{low} cell lines did not demonstrate the combined effects of both drugs. Therefore, the EpCAM^{high} cell lines were chosen for further analysis.

Sphere Formation Assay

After the treatment with VB4-845 and/or 5-FU on EpCAM^{high} cell lines, the viable cells were collected and analyzed for their ability to form spheres on reculturing (Fig. 2a). After 7 days, the number of spheres (>100 μm in diameter) was significantly decreased after exposure to VB4-845 alone or the combination in all of the four cell lines, but not altered after exposure to 5-FU alone (Fig. 2b). Although the doses of VB4-845 and 5-FU used in this assay showed similar antiproliferative activity

(Fig. 1d), their effects on sphere-forming ability were in direct opposition to one another. Because the sphere-forming cells are assumed to be capable of self-renewal, one of essential hallmarks of stemness, the effect of VB4-845 for EpCAM^{high} cell lines was found to be closely associated with their stemness.[25,29]

Alterations of Stem/Progenitor Markers

In the EpCAM^{high} cell lines, we analyzed the expression of several stem/progenitor markers, such as CD133, CD13, CD44, and CD90, using FACS analysis after the treatment of VB4-845 and/or 5-FU (Fig. 3a). These markers were chosen, because they were reported as biomarkers of HCC with poor prognosis.[7–10] The positive rate of CD133 was significantly decreased with the treatment of VB4-845 or combination, but increased with the treatment of 5-FU (Fig. 3b; $p < 0.05$). Interestingly, HepG2 cells showed a unique bimodal pattern for CD133 expression (Fig. 3a, arrow) and VB4-845 dramatically decreased this CD133-positive subpopulation of HepG2 cells with statistical significance. The positive rate of CD13 was significantly decreased with VB4-845, but increased with 5-FU treatment in HuH-7 and Hep3B cells (Fig. 3c, $p < 0.05$). There was no consistent tendency for the positive rate of CD44 and CD90 after the treatment of each cell line. Our results might indicate the effect of VB4-845 might be closely associated with the stemness of human HCC cells.

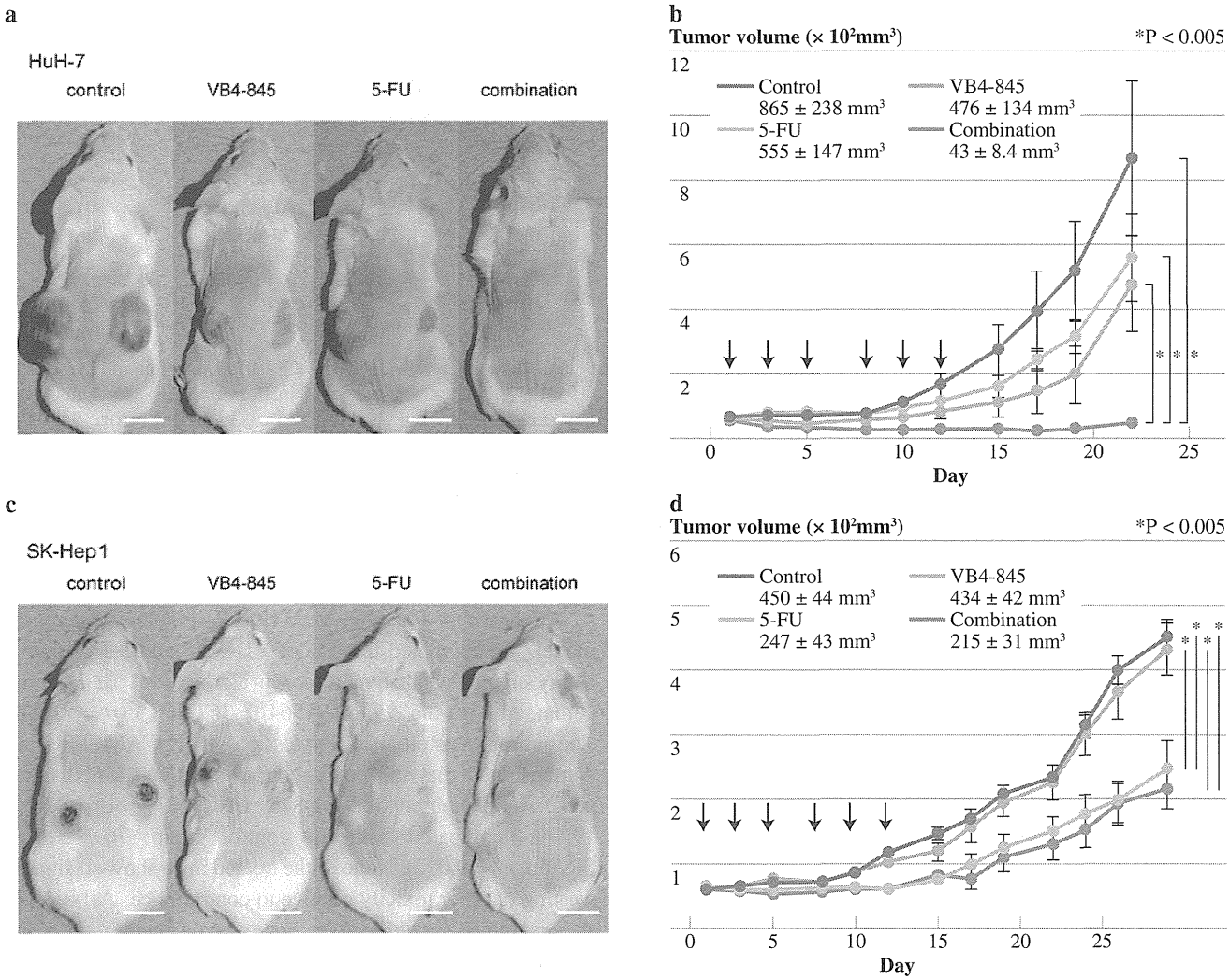


FIG. 4 In vivo studies in subcutaneous xenograft models using HuH-7 EpCAM^{high} or SK-Hep1 EpCAM^{low} HCC cells. Established subcutaneous xenografts derived from HuH-7 (**a**, **b**) or SK-Hep1 (**c**, **d**) were treated with intravenous injection of control saline or VB4-845 (30 μg/kg) and intraperitoneal injection of control saline or 5-FU (30 mg/kg) three times per week for 2 weeks. **a**, **c** Representative subcutaneous tumors in mice at the end of the dosing period. *Scale bar*, 10 mm. **b**, **d** Tumor size were measured using calipers three times a week in the four groups, and volumes were calculated using the following equation: volume = (length) × (width)^2 × 0.5. *Arrows* indicate the time of administration. *Vertical bars*, standard error. Statistical analysis was done by two-tailed Student's *t* test ($p < 0.05$)

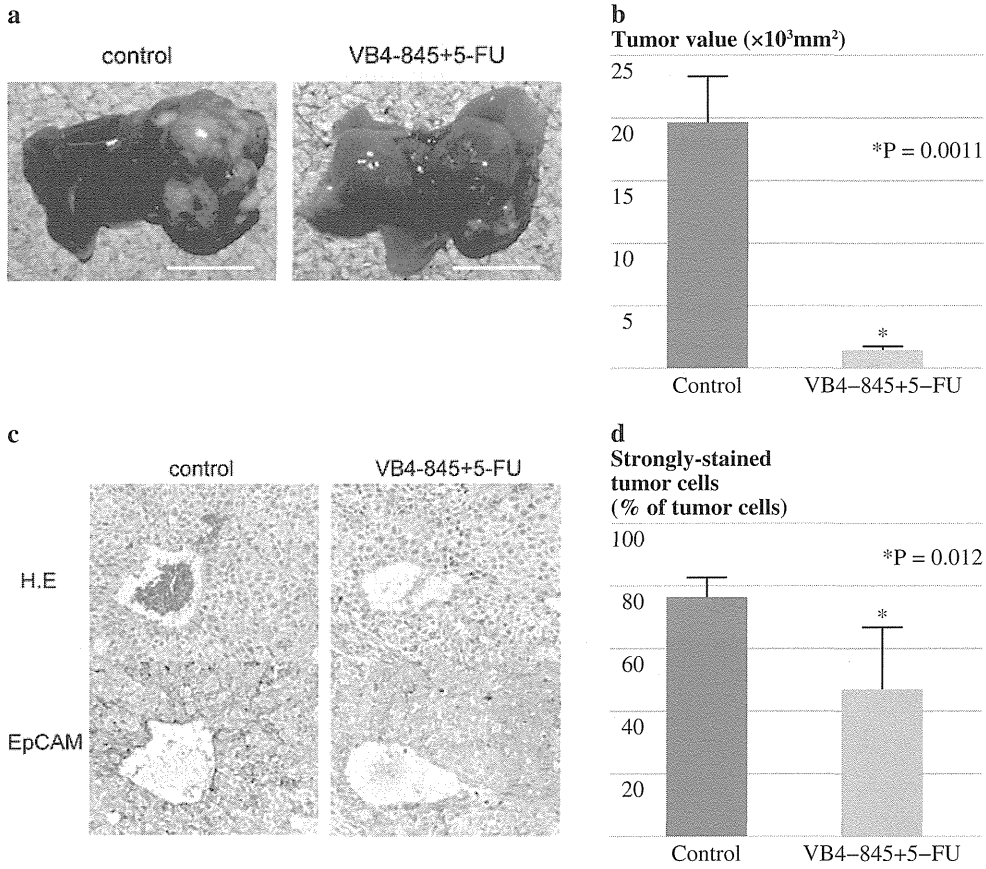


FIG. 5 In vivo studies in liver orthotopic xenograft models. Established liver orthotopic xenografts of HuH-7 were treated with control saline or the combination of VB4-845 plus 5-FU. The method and schedule of administration was the same as the subcutaneous tumor. Two weeks after the initiation of treatment, mice were sacrificed and the size of liver tumor was measured. **a** Representative liver tumor in mice at the end of the dosing period. *Scale bar*, 10 mm. **b** Liver tumor volume was analyzed 2 weeks after administration of the control (1964 ± 367 mm^3) or the combination of VB4-845 plus 5-FU (141 ± 34 mm^3) ($n = 5$). *Vertical bars*, standard error. Statistical analysis was done by two-tailed Student's t test ($p = 0.0011$). **c** H&E staining and immunostaining of EpCAM (magnification, ×40). **d** The percentage of strongly stained tumor cells in all of tumor cells between two groups. *Vertical bars*, standard deviation

In Vivo Effects of VB4-845 and/or 5-FU

To investigate in vivo antitumor activity, NOD.CB17-PRkdc^{Scid}/J mice bearing established HuH-7 (EpCAM^{high}) and SK-Hep1 (EpCAM^{low}) subcutaneous xenografts were utilized. In HuH-7 xenograft model, the volume of the tumors in the VB4-845 and 5-FU monotherapy groups appeared smaller, albeit not significantly, compared with the control group ($p = 0.078$ and 0.31, respectively, Figs. 4a, b). It is noteworthy that significant tumor regression was observed in the group treated with VB4-845 plus 5-FU compared with either the control or monotherapy groups ($p < 0.005$). On the other hand, any antitumor effect was not observed in SK-Hep1 EpCAM^{low} xenografts by treatment with VB4-845 monotherapy nor combination therapy (Figs. 4c, d). None of the treated mice showed signs of wasting or other toxicity relative to control mice.

As shown in Figs. 5a, b of HuH-7 orthotopic xenograft model, the combined therapy of VB4-845 and 5-FU significantly suppressed the liver tumors in all mice (141 ± 34 mm^3) compared with the control (1964 ± 367 mm^3) ($p = 0.011$). The immunohistological expression of EpCAM (Fig. 5c) demonstrated that the population of strongly-stained tumor cells was decreased in VB4-845 plus 5-FU group (47.4 ± 19.4 %) compared with the control group (76.7 ± 6 %; Fig. 5d, $p = 0.012$). None of the treated mice showed signs of wasting or other toxicity relative to control mice. All host tissues examined, including liver, bone marrow, kidney, intestine, and lung, were histologically normal in all experiments.

DISCUSSION

In this study, our FACS analysis of EpCAM expression revealed that eight human HCC cell lines were classified

into two groups: four $EpCAM^{high}$ (>95 %) and four $EpCAM^{low}$ (<5 %) HCC (Fig. 1a). This is supported by the fact that VB4-845 was exceedingly effective against $EpCAM^{high}$ cell lines but not $EpCAM^{low}$ cell lines (Figs. 1b, d). Because the close correlation between EpCAM expression and sphere-formation was reported in HCC cells, we assessed the effect of VB4-845 on sphere-forming ability.[11] Although 5-FU treatment did not affect the sphere formation, the treatment with VB4-845 as well as the combination of VB4-845 plus 5-FU clearly suppressed the sphere formation in all four HCC cell lines (Figs. 2a, b; $p < 0.001$). Because the sphere-forming ability is known to be regulated by the self-renewing capacity of stem cells, the effects of VB4-845 might be closely associated with the stemness of $EpCAM^{high}$ HCC cells.[25,29]

For further investigation of the VB4-845 effects on the stemness, the expression of several stem/progenitor markers was analyzed after the treatment of VB4-845 and/or 5-FU. 5-FU treatment significantly increased the positive rates of CD133 in threeHCC cell lines (Fig. 3b; $p < 0.05$). Ma et al.[30] reported that $CD133^+$ subpopulation in HCC cells was more resistant to 5-FU than $CD133^-$ subpopulation, suggesting the $CD133^+$ subpopulation might contribute to the chemoresistance of HCC. Furthermore, VB4-845 dramatically decreased the $CD133^+$ subpopulations in these HCC cells (Fig. 3b; $p < 0.005$). Similar results were obtained from the analysis of CD13. The positive rates of CD13 were significantly decreased by VB4-845 treatment but increased by 5-FU treatment (Fig. 3c). These results indicated that the targeted subpopulations were different between the VB4-845 and 5-FU treatments. With respect to the stem/progenitor markers, the effects of VB4-845 also were found to be closely associated with the stemness of human HCC cells.

Because in vitro cytotoxic effects of VB4-845 and/or 5-FU were observed with or without apoptosis (Fig. 1d and Supplementary Fig. 2), in vivo antitumor effects were analyzed using the subcutaneous xenograft model (Fig. 4). Whereas a statistically significant antitumor effect was not detected by either VB4-845 or 5-FU monotherapy, the combination therapy of VB4-845 and 5-FU significantly decreased the exotopic tumors of $EpCAM^{high}$ HCC ($p < 0.05$). Because the organ microenvironment in cancer might play a critical role in drug sensitivity, particularly for HCC, an organotropic cancer, a liver orthotopic xenograft model having similarity with the clinical condition also was utilized to explore tumor growth inhibition.[31] As observed in the subcutaneous xenograft model, significant regression of tumors was observed in the VB4-845 plus 5-FU treated group compared with the control group (Figs. 5a, b; $p = 0.0011$).

We have previously reported that the EpCAM expression was significantly associated with poor prognosis of the patients with in CM-type HCC.[17] Indeed, our preliminary prospective study validated EpCAM as the predictive biomarker of the patient prognosis ($p = 0.0447$) as well as the recurrence (Supplementary Fig. 3, $p = 0.0171$). Other clinical studies indicated that EpCAM was one of the biomarkers of chemoresistance in HCC. Noda et al.[32] reported the correlation between the EpCAM expression and chemoresistance to interferon-α/5-FU combination therapy for HCC. In another report, the expression of EpCAM and CD133 in chemoembolized HCC tumor cells was significantly correlated with the HCC recurrence after liver transplantation.[33] These findings indicate the cancer cells expressing EpCAM could be an important target in the treatment of refractory HCC.

In our studies of VB4-845, the EpCAM-targeted therapy appears to demonstrate anticancer effects via potentially different mechanism (e.g., stemness) from the conventional cytotoxic agent 5-FU. Indeed, our preclinical studies intimate that the combination therapy of an immunotoxin targeting EpCAM with a conventional cytotoxic agent is a promising novel approach for the treatment of human HCC. Further studies and clinical trials of EpCAM-targeting agents will confirm its therapeutic role in the HCC management.

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CONFLICT OF INTEREST The authors declare no competing financial interests.

DISCLOSURES The authors disclose no conflicts.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Mitochondrial metabolism in the noncancerous liver determine the occurrence of hepatocellular carcinoma: a prospective study

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Abstract

Background Recurrence determines the postoperative prognosis with hepatocellular carcinoma (HCC). It is unknown how the liver dysfunction involving organic anion transporter failure causes the occurrence of HCCs. This study was designed to elucidate the link between liver dysfunction and multicentric occurrence (MO) after radical hepatectomy.

Methods Forty-nine samples of noncancerous liver tissue from HCC patients within the Milan criteria who were treated at our institution between January 2004 and August 2008 were examined as a training set by using genome-wide gene expression analysis. Using the independent 2-institutional cohort of 134 patients between September 2008 and December 2009, we performed a validation study using tissue microarray analysis. Cox proportional hazard regression analyses for MFS were performed to estimate the risk factors.

Results In the Gene Ontology database (GO:0015711), SLC22A7 expression was the best predictor of MO-free survival [MFS] (Fold, 0.726; $P = 0.001$). High SLC22A7 gene expression prevented the occurrence of HCC after hepatectomy (odds ratio [OR], 0.2; $P = 0.004$). Multivariate analyses identified SLC22A7 expression as an independent risk factor (OR, 0.3; $P = 0.043$). In the validation study, multivariate analyses of MFS identified SLC22A7 expression as an independent risk factor (OR, 0.5; $P = 0.012$). As judged by gene set enrichment analysis, SLC22A7 down regulation was associated with mitochondrion ($P = 0.008$) and oxidoreductase activity ($P = 0.006$). Sirtuin 3 as a regulator of mitochondrial metabolism also determined MFS ($P = 0.018$).

Conclusions The mitochondrial pathways may affect SLC 22A7 function to promote the occurrence of HCC. (Word count: 246).

Keywords Hepatocellular carcinoma · Mitochondria · Sirtuin 3 · SLC22A7 · Organic anion transporter

Abbreviations

CI	Confidence interval
FDR	False discovery rate

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GSEA Gene set enrichment analysis
HCC Hepatocellular carcinoma
HR Hazard ratio
MO Multicentric occurrence
NES Normalized enrichment score
OR Odds ratio

Introduction

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related deaths worldwide because of its high fatality (overall ratio of mortality to incidence of 0.93) [1]. In 2008, an estimated 748,000 new cases of HCC and 696,000 deaths associated with HCC occurred [1]. Underlying liver diseases increase the risk of HCC occurrence, although the mechanism by which this occurs is unclear [2]. Multicentric occurrence (MO) is a crucial problem irrespective of anatomic resection to prevent local recurrences. The clinical courses and biological features of MO definitely differ from those of local recurrence and intrahepatic metastases [3, 4].

Various treatments are selected for MO of HCC [5]. Anatomic resection proposed by Makuuchi et al., was implemented to overcome the local recurrence involving micro-dissemination into the portal vein and intrahepatic metastasis [6]. Resection is regarded as a first-line therapy when HCC occurrence is within the Milan criteria. However, even after curative treatments involving anatomic resection, considerable risk of MO has been reported [7]. There is no benefit of anatomic resection in HCC with MO [8]. Noncancerous liver tissue with oncogenic potential may explain the risk of MO after hepatectomy [3]. The criteria for MO are defined in the classification of the Liver Cancer Study Group of Japan [4].

This study was designed to elucidate whether noncancerous liver function involving transporter activity influences the MO of early-stage HCC. This study excluded patients beyond the Milan criteria to reduce malignant factors of the primary tumor. Genome-wide gene expression analysis was used to elucidate the link between this hepatocellular function and MO of HCC. Hepatocellular organic anion transporters exchange materials that are indispensable for mitochondrial metabolism, and they detoxify the sinusoidal microcirculation. Xenobiotics transported through organic anion transporters, are detoxified in hepatocytes and excreted into bile [9]. In the organic anion transporter genes according to the Gene Ontology database (GO:0015711) as a hepatocellular function, the best predictor for MO of HCC was SLC22A7. According to recent reports, SLC22A7 expressed on the hepatocellular sinusoidal membrane takes up orotic acid [10, 11]. An experimental study reported that exposure to dietary orotic acid with hepatectomy promotes liver carcinogenesis [12]. In this study, we present evidence indicating that decreased SLC22A7 expression associated with mitochondrial disability might play a causative role in liver carcinogenesis and that it will be a biomarker for predicting MO even after curative hepatic resection.

Methods

Training set

Between January 2004 and August 2008, 231 curative hepatectomies for HCC were performed at Tokyo Medical and Dental University Hospital (Tokyo, Japan). In total, 69 of 115 patients within the Milan criteria were ethically informed according to the guidelines of our institutional review board. This study excluded "beyond Milan", a contraindication for anatomic resection and liver transplantation. Trans-arterial embolization, radiofrequency ablation, and systemic chemotherapy are available options when tumor recurrence is evident after the first hepatectomy. Serum alpha-fetoprotein (AFP) and des-gamma-carboxy prothrombin (DCP) levels were measured monthly, and ultrasonography, computed tomography, and magnetic resonance imaging were performed every 3 months. The criteria for MO of HCC were defined according to the classification of the Liver Cancer Study Group of Japan (the recurrent tumor consists of early HCC occurring in a different hepatic segment with or without dysplastic nodules in peripheral areas, or well differentiated HCCs with peripheral moderately or poorly differentiated HCC) [4]. Any tumor, regardless of the time to recurrence, arising in the same segment as the initial tumor (or within 2 cm from the surgical stump when performing segmentectomy) was considered a "local" recurrence [13].

Genome-wide gene expression analysis

All samples of noncancerous liver tissue obtained from the resected specimens were separately frozen immediately and stored at −80 °C. Total RNA was extracted using an RNeasy kit (Qiagen, Hilden, Germany). Contaminating DNA was removed by digestion with RNase-free DNase (Qiagen). Upon checking the RNA integrity of the samples using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), 49 samples were given an RNA integrity number exceeding 5.0. After preparing complementary RNA by 1-cycle target labeling and with a control reagents kit (Affymetrix, Santa Clara, CA, USA), hybridization and signal detection of HG-U133 Plus 2.0 arrays (Affymetrix) were performed according to the manufacturer's instructions. The 49 microarray datasets were normalized using the robust multiarray average method of R

statistical software (version 2.12.1) together with the Bio-Conductor package. Estimated gene expression levels were obtained as log2-transformed values, and 62 control probe sets were selected for further analysis.

Selection of organic anion transporter genes for HCC occurrence

First, probe sets corresponding to known genes were selected on the basis of the NetAffx annotation file, version 32 (available at: http://www.affymetrix.com/analysis/index.affx). Next, probe sets of organic anion transporter genes were selected according to GO:0015711 (52 probes). The 35 probes were matched to the criteria. The univariate Cox proportional hazards regression model was used to estimate the relationship between the gene expression pattern and MO for each probe set. Probe sets that had a $P < 0.005$ by the likelihood ratio test were selected.

Validation study on immunohistochemical analysis using tissue microarrays

To validate the clinical significance of SLC22A7 expression, 134 patients who visited Tokyo Medical and Dental University Hospital and Nihon University Hospital between September 2008 and December 2009 were enrolled in the prospective multicenter cohort. The candidate gene was assessed by immunohistochemical staining on tissue microarrays using resected liver samples from patients with HCC within the Milan criteria with an anti-SLC22A7 antibody (provided by Dr. Anzai) at a 1:20 dilution [14] by the use of an automated immunostainer (Ventana XT System; Ventana Medical Systems, Inc., Tucson, AZ, USA) as described previously. The SLC22A7 staining was judged by 2 investigations, and staining of less than 25 % of cells was judged as negative (Fig. 1c, d).

Gene set enrichment analysis (GSEA)

To investigate the biological backgrounds correlated with a particular gene expression pattern, we used GSEA version 2.0.7 with MSigDB gene sets version 3.0. Gene set category C5, which is based on the GO database, was used. Gene sets satisfying both $P < 0.05$ and a false discovery rate (FDR) <0.25 were considered significant. The customized sirtuin 3-related gene set involving 147 genes was constructed to examine the relationship with SLC22A7 according to the supplemental Fig. 1.

Statistical analysis

Univariate and multivariate Cox regression analyses were performed using SPSS 20.0 (IBM, Armonk, NY, USA). The median value was selected for the cut-offs of clinical variables in the training set and the validation set. The MO-free survival (MFS) was evaluated by the Kaplan–Meier method and the log-rank test. Two-sided $P < 0.05$ were considered significant. Values are given as the mean ± SD unless otherwise stated.

Results

Baseline characteristics

All possible curative resections within the Milan criteria (R0) were attempted for the 49 patients in the training set and the 134 patients in a multicenter validation study (Table 1). The mean observation time in the training set and the validation set were 21.1 and 16.4 months, respectively. The mean MFS in the training set and the validation set were 12.3 and 10.6 months. There was no difference in the mean MFS between the training and validation sets ($P = 0.602$), though the mean observation time was longer in the training set ($P = 0.010$). There was no difference between the two studies in age, gender, viral infection (HBV and HCV), serum albumin, total bilirubin, AFP, DCP, platelet count, tumor number, pathological invasion into the portal vein, liver cirrhosis, and MO occurrence rate. There were differences in Child class B ($P = 0.03$), ICG-R15 value ($P = 0.02$), tumor size ($P < 0.0001$), and anatomic resection ($P < 0.0001$), respectively.

The predictive factors for MFS in the training set

As shown in Table 2, low SLC22A7 expression was the best predictor of MFS ($P = 0.001$; fold difference between the mean expression levels of patients with and without MO = 0.726). Table 3 presents the link between MFS and SLC22A7 gene expression in the 49 HCC patients within the Milan criteria. The MO was observed in seventeen patients (35 %). Univariate analyses identified HCV infection, serum albumin levels, serum platelet counts, and SLC22A7 gene expression as risk factors for HCC occurrence. These results led us to determine which risk factors were independently predictive of the prognosis of HCC patients. According to multivariate analysis, SLC22A7 gene expression determined prognosis independently. Figure 1a indicates that the cumulative recurrence-free survivals were significantly associated with SLC22A7 expression. (Log-rank test, $P = 0.001$).

Validation study for SLC22A7 expression among patients within the Milan criteria

Table 4 illustrates the link between MFS and SLC22A7 protein expression as judged by a tissue microarray in 134

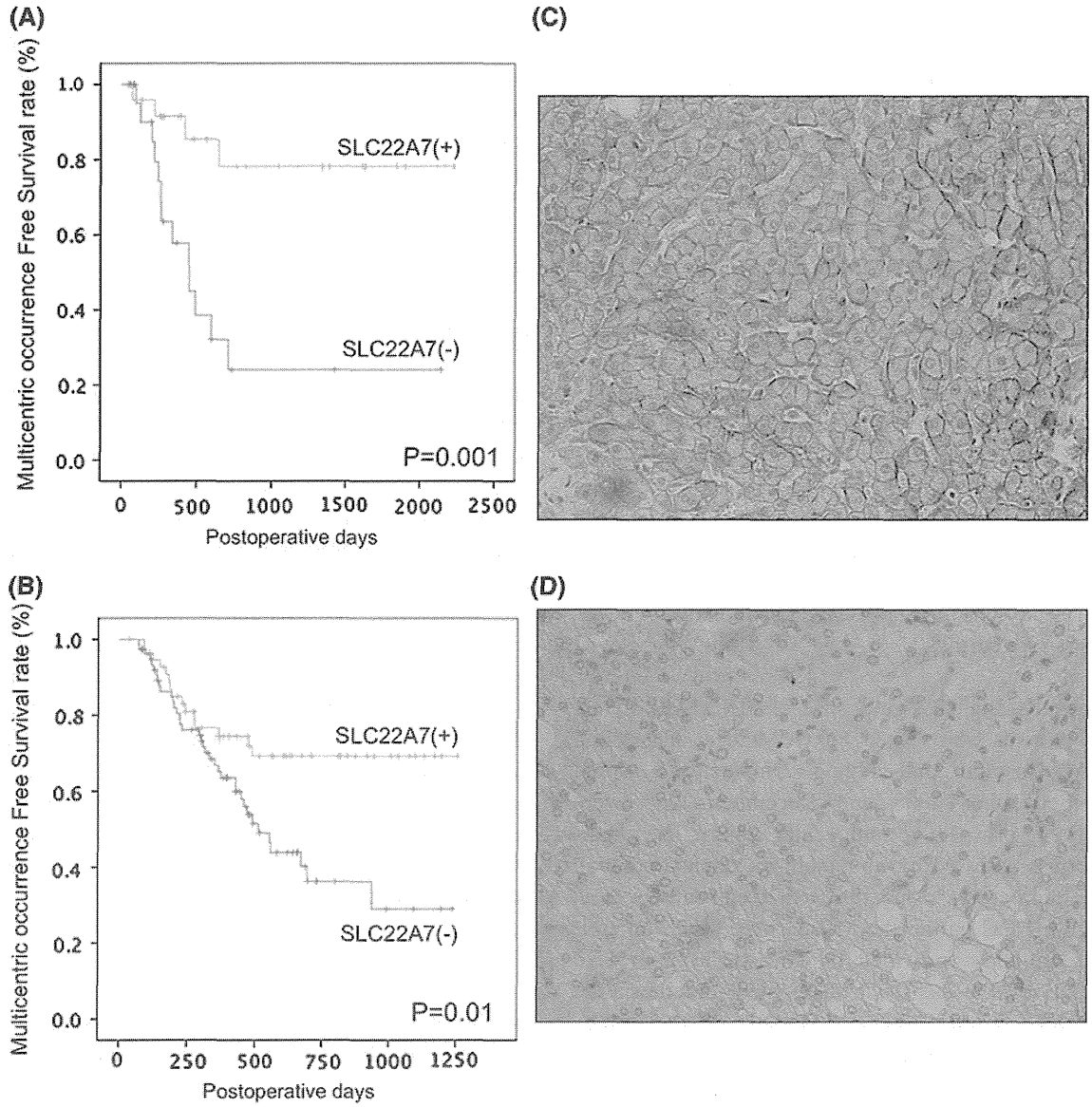

Fig. 1 **a** Training study. MFS of postoperative HCC patients within the Milan criteria with high (SLC22A7 (+) group) and low (SLC22A7 (−) group) SLC22A7 gene expression in noncancerous liver tissue. The median expression level for each gene was used as a cutoff value. The green lines denote the Kaplan–Meier curves for the SLC22A7 (+) group. *Blue line* denotes low SLC22A7 expression in the SLC22A7 (−) group. The prognosis of the SLC22A7 (−) group was significantly worse ($P = 0.001$). **b** Validation study using a prospective multicenter cohort. The *green lines* denote the Kaplan–Meier curves for SLC22A7 (+) group. The *blue line* denotes the MFS in the SLC22A7 (−) group. Note the poor survival of the SLC22A7 (−) group ($P = 0.01$). **c** Immunohistochemical analysis (magnification, ×20) with high expression of SLC22A7. **d** Immunohistochemical analysis (magnification, ×20) with low expression of SLC22A7 protein

HCC patients within the Milan criteria. The SLC22A7 protein was expressed at the hepatocellular sinusoidal membrane in noncancerous tissues (Fig. 1c). The MO was observed in 52 patients (38 %). Low SLC22A7 expression was confirmed when the immunohistochemical staining of the tissue microarray was less than 25 % (Fig. 1d). Univariate analyses identified anatomic resection and low SLC22A7 expression as risk factors for HCC occurrence (Table 4). Other clinicopathological factors did not determine MO. These results led us to determine which factors were independently predictive of prognosis. As shown in Table 4, SLC22A7 expression determined prognosis (OR, 0.5; 95 % CI, 0.3–0.8; $P = 0.012$). In Fig. 1b, the cumulative MFS was significantly associated with SLC22A7 expression ($P = 0.010$). The 1-year MFS in patients with low SLC22A7 expression was 65.2 %, compared with 76.7 % in those with high SLC22A7 expression.

GSEA evaluation of SLC22A7 expression in HCC

The dataset had 54,675 native features. After collapsing the features into gene symbols, 20,606 genes were identified.

Gene set size filters (min = 15, max = 500) resulted in the filtering out of 446/1454 gene sets. The remaining 998 gene sets containing 7,605 genes were used in the analysis. The p value of SLC22A7 was ranked at 261st out of 21,050 genes included in the HG-U133 Plus 2.0 array. In total, 552 of 998 gene sets were positively correlated with SLC22A7 expression. Seventy-seven gene sets were significant at FDR of <25 %. Thirty-six gene sets were significantly enriched at a nominal $P < 1$ %. Conversely, 446 of 998 gene sets were negatively correlated with SLC22A7 expression. No gene sets were significantly enriched at FDR < 25 %. Two gene sets were significantly enriched at a nominal $P < 1$ %. As shown in Fig. 2, mitochondrion ($P = 0.008$; FDR = 0.199; NES = 1.804), oxidoreductase activity ($P = 0.006$; FDR = 0.157; NES = 1.854), and fatty acid metabolic process ($P = 0.021$; FDR = 0.177; NES = 1.723) were significantly correlated with SLC22A7 expression. By analyzing the gene expression profiles of 49 samples of noncancerous tissue, GSEA showed that the 27 of the 62 gene sets (44 %) were closely related with mitochondrial genes involving oxidoreductase activity and fatty acid metabolic process at FDR of 20 % with a nominal $P < 0.05$ (Supplementary Table 1). Mitochondrial sirtuin 3, reported as the regulator of fatty acid oxidation, oxidative damage and orotic acid concentrations, prevents deacetylates and stimulates ornithine transcarbamylase (OTC) and modulates amino acid catabolism and β-oxidation [15, 16]. The correlation between SLC22A7 and sirtuin3 expression levels was 0.300 ($P = 0.034$). As shown in Fig. 2d, decreased sirtuin 3 gene expression also determined patient MFS ($P = 0.018$). These results led us to examine whether the customized sirtuin 3-related gene set correlates with SLC22A7. The GSEA revealed a remarkable correlation with SLC22A7 ($P = 0.008$; FDR = .008; NES = 1.786), as shown in Supplemental Figs. 2 and 3. Mitochondrial factor may be confounding factor of the two factors, though the detailed mechanism remains unknown.

Table 1 Baseline characteristics

Variables	Training set Mean (SD)	Validation set Mean (SD)	P
Age	66.8 ± 10.3	67.3 ± 9	0.941
Male/female	34/15	97/37	0.411
Viral infection			
HBV	11	17	0.136
HCV	31	85	0.363
Laboratory data			
Prothrombin time (%)	83.7 ± 17.9	92.3 ± 12.8	<0.0001*
Albumin (g/dL)	3.9 ± 0.5	4 ± 0.5	0.067
Total bilirubin (mg/dL)	0.8 ± 0.4	0.8 ± 0.4	0.401
Platelet ($\times 10^4$/mL)	14.1 ± 6.8	14.4 ± 5.6	0.509
Child-Pugh A/B	42/7	128/6	0.03*
ICG-R15 (%)	20.2 ± 11.9	16.3 ± 10.2	0.015*
Tumor factor			
Diameter (cm)	3.2 ± 1	2.5 ± 1	<0.0001*
Number	1.4 ± 0.9	1.2 ± 0.5	0.159
AFP (ng/mL)	350 ± 1305	175 ± 750	0.272
DCP (mAU/mL)	1053 ± 3389	1345 ± 7086	0.958
Pathological vp (+)	9	22	0.790
Anatomic resection			
Yes	24	28	<0.0001*
Liver background			
NL/CH/LC	3/17/29	6/71/57	0.081
Multicentric occurrence			
+	17	52	0.371

DCP des-gamma-carboxy prothrombin

* $P < 0.05$ was considered significant

Discussion

The retrospective training study and validated prospective multicenter study provided evidence that low SLC22A7 expression promoted the occurrence of HCC after hepatectomy in patients within the Milan criteria. This is the first study to elucidate the link between the occurrence of HCC and SLC22A7 expression in noncancerous liver

Table 2 The univariate analyses to estimate the relationship between a gene expression pattern and MO of HCC for each probe set of organic anion transporter genes according to the Gene Ontology database (GO:0015711)

Probe set	Symbol	Title	Fold	P
221661_at	SLC22A7	Solute carrier family 22 (organic anion transporter), member 7	0.726	0.001*
1557918_s_at	SLC16A1	Solute carrier family 16, member 1 (monocarboxylic acid transporter 1)	0.831	0.005
210366_at	SLC10A1	Solute carrier family 10 (sodium/bile acid cotransporter family), member 1	0.936	0.017
207185_at	SLC16A1	Solute carrier family 16, member 1 (monocarboxylic acid transporter 1)	0.907	0.071
202236_s_at	SLCO1A2	Solute carrier organic anion transporter family, member 1A2	0.912	0.074

The best organic anion transporter genes in GO-0015711. † Fold values were calculated by the ratio of mean expression levels in the patients with MO to that in patients without MO

* $P < .005$ was considered significant (Cox's proportional hazard ratio)

Table 3 The risk factors determining MO in 49 HCC patients within the Milan criteria (training set)

Variables	Univariate		*P*	Multivariate		*P*
	OR	95 %CI		OR	95 %CI	
Age (years)						
>68	1.7	(0.6–4.4)	0.288			
Gender						
Female	1.2	(0.5–3.3)	0.694			
HCV						
(+)	0.2	(0.1–0.9)	0.027*	0.4	(0.1–1.7)	0.206
HBV						
(+)	2.7	(0.6–11.9)	0.190			
Total bilirubin (×mg/dL)						
≧0.8	1.9	(0.7–5.5)	0.241			
Albumin (g/dL)						
≧4.0	0.2	(0.1–0.6)	0.002*	0.3	(0.1–1.0)	0.056
Prothrombin time (%)						
≧84.4	0.7	(0.3–1.8)	0.426			
Platelet ($\times 10^4/\mu L$)						
≧11.9	0.3	(0.1–0.8)	0.017*	0.8	(0.2–2.8)	0.700
Child-Pugh A vs. B	0.4	(0.1–1.4)	0.140			
ICG-R15 (%)						
≧20	2.3	(0.9–6.1)	0.101			
Tumor diameter (cm)						
>3	0.9	(0.4–2.4)	0.871			
Multiple	1.6	(0.5–5.7)	0.444			
AFP (ng/mL)						
≧12	1.1	(0.9–1.3)	0.337			
DCP (mAU/mL)						
≧38	0.6	(0.2–1.6)	0.284			
Capsule						
(+)	0.4	(0.0–20)	0.309			
Capsule invasion						
(+)	0.5	(0.2–1.3)	0.151			
Pathological vp						
(+)	0.9	(0.2–4.0)	0.909			
CM type vs. SNEG	1.0	(0.3–3.5)	0.972			
SN type vs. SNEG	0.3	(0.1–1.1)	0.061			
Moderately differentiated						
Vs. well	0.8	(0.2–3.5)	0.775			
Poorly differentiated						
Vs. well	0.2	(0.1–2.0)	0.233			
Liver cirrhosis						
(+)	1.7	(0.6–4.6)	0.298			
SLC22A7 expression						
High	0.2	(0.1–0.6)	0.004*	0.3	(0.1–1.0)	0.043*
Anatomic resection						
(+)	0.7	(0.3–1.8)	0.426			

DCP des-gamma-carboxy prothrombin *SN* simple nodular type, *SNEG* simple nodular type with extranodular growth, *MC type* confluent multinodular type

* $P < .05$ was considered significant

tissue. Moreover, this gene expression is closely related with the gene sets of mitochondrion in noncancerous liver, as judged by GSEA evaluation in the training set (Fig. 2a).

Table 4 The risk factors that determine MO in 134 HCC patients within the Milan criteria (validation set)

Variables	Univariate		*P*	Multivariate		*P*
	OR	95 %CI		OR	95 %CI	
Age						
≧67	1.08	(0.62–1.89)	0.792			
Gender						
Female	0.88	(0.46–1.68)	0.701			
HCV						
(+)	1.13	(0.64–1.98)	0.681			
Body weight						
≧58.3	1.00	(0.58–1.74)	0.989			
Total bilirubin (mg/dL)						
≧0.7	0.79	(0.45–1.39)	0.418			
Albumin (g/dL)						
≧4.1	0.70	(0.40–1.21)	0.200			
Prothrombin time (%)						
≧97 %	0.79	(0.46–1.37)	0.406			
Platelet ($\times 10^4/\mu L$)						
≧13.8	0.80	(0.46–1.37)	0.413			
Child-Pugh score						
≧6	0.81	(0.41–1.62)	0.550			
ICG-R15 (%)						
≧14.3	1.17	(0.68–2.03)	0.568			
Tumor diameter (cm)						
≧2.4	0.88	(0.51–1.52)	0.644			
Multiple tumor						
(+)	1.28	(0.57–2.84)	0.550			
Pathological vp						
(+)	0.99	(0.48–2.04)	0.985			
AFP (ng/mL)						
≧8.7	1.41	(0.82–2.50)	0.216			
DCP (mAU/mL)						
>36	0.75	(0.43–1.30)	0.302			
Liver cirrhosis						
(+)	0.70	(0.21–2.33)	0.558			
Anatomic resection						
(+)	0.52	(0.24–1.16)	0.110			
SLC22A7 expression						
High	0.46	(0.25–0.84)	0.012*	0.46	(0.25–0.84)	0.012*

DCP des-gamma-carboxy prothrombin

* $P < 0.05$ was considered significant

The GSEA showed that the 44 % of SLC22A7-related gene sets were closely related with mitochondrial genes involving oxidoreductase activity and fatty acid metabolic process. Mitochondrial metabolism may also involved fatty acid synthase and oxidoreductase activity.

A previous study demonstrated that the gene expression profiles of the surrounding nontumoral liver tissue, but not the tumor tissues, were highly correlated with survival in the training set of Japanese patients and in validation sets in the United States and Europe ($P = 0.04$) [17]. Anatomic resection was not identified as a prognostic factor in the two studies. The HCV infection, platelet counts, and serum