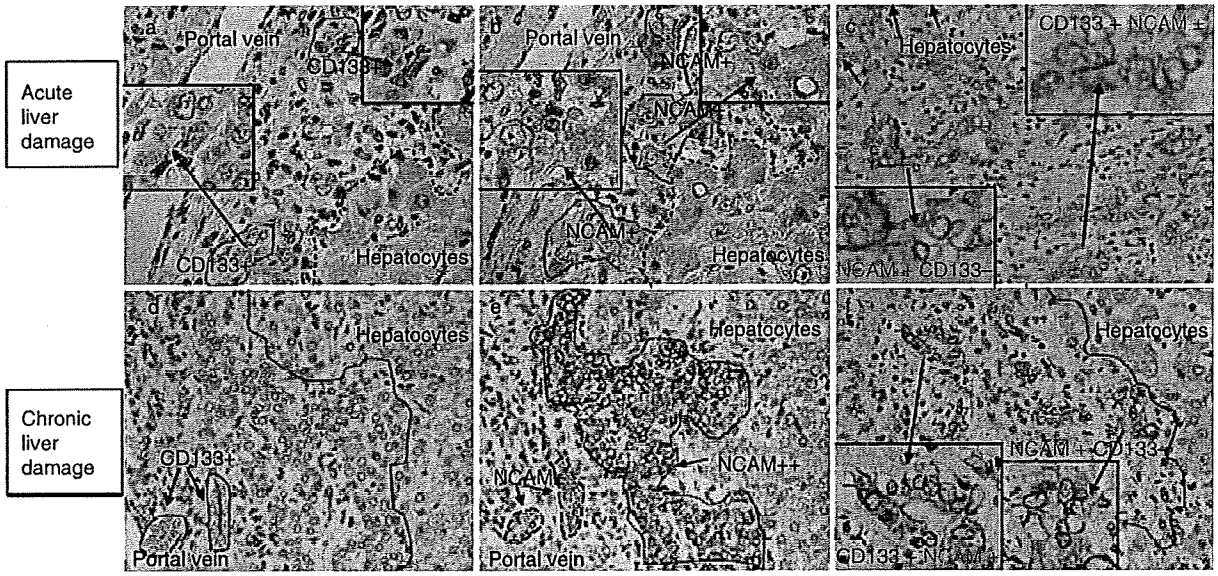

Figure 4 Analysis of the distribution of CD133⁺ cells and neural cell adhesion molecule (NCAM)⁺ cells in ductular reactions (DR). Serial section analysis of acute damaged livers (a,b) and chronically damaged livers (d,e). Double immunohistochemistry analysis of acute damaged liver (c) and chronically damaged liver (f). NCAM⁺ cells distributed near the hepatocytes can be seen in both acute and chronically damaged livers. Double immunohistochemistry analysis revealed the presence of CD133⁺NCAM⁻, CD133⁺NCAM⁺ and CD133⁻NCAM⁺ DR in acute and chronically damaged livers. CD133⁻NCAM⁺ DR were always found relatively nearer to the hepatocytes than either CD133⁺NCAM⁻ DR or CD133⁺NCAM⁺ DR. Similar results were seen in other patients. Original magnification × 200. (a,b) Case 12 in Table 1; (c) case 7 in Table 1; (d,e) case 22 in Table 2; (f) case 12 in Table 2.

livers. Because serial section and double staining analysis revealed that CD133⁻NCAM⁺ cells were mainly located between CD133⁺ cells and CPSI⁺ mature hepatocytes, and consisted of cells that were morphologically similar to hepatocytes, we speculated that NCAM⁺ cells include those cells that are at a relatively mature stage in the hepatocytic lineage compared to CD133⁺ cells. The relationship between CD133⁺ cells and NCAM⁺ cells was common to both acute and chronically damaged livers.

CD133⁺ and NCAM⁺ cells became more apparent in proportion to the increase in their levels of fibrosis and histological damage. While CD133⁺ cells and NCAM⁺ cells were rarely detected below stages F1/A1, they began to proliferate in liver tissue graded as stages F2/A2 and above. Furthermore, cells expressing these markers proliferated extensively in livers that had sustained further damage, due to portal vein thrombosis or treatment by TACE. An increase in the AFP serum level can often be seen following acute or chronic damage,[33] although in the cases in our study, there was no remarkable elevation in AFP levels in NCAM and CD133 expanded cases, nor was there any elevation in the serum levels of ALT. These results indicate that serum levels of AFP and ALT do not necessarily mirror the proliferation of CD133⁺ cells and NCAM⁺ cells.

CD133 is expressed at the apical regions of DR. While the role of CD133 has remained unclear, it has been recently proposed as a candidate cancer stem cell marker in cell line studies.[19–21] In our investigation, while CD133 (AC133) was not detected in HCC tissue itself, CD133⁺ cells were detected in regions of fibrosis surrounding the tumor tissue. Our results suggest that, for most HCC, if CD133⁺ cells do exist, they are very rare and could not be detected by our methods. However, before a final conclusion can be reached as to whether CD133 is a useful cancer stem cell marker for HCC, several problems have to be resolved. For instance, it is possible that the AC133 epitope might be masked under certain circumstances such as glycosylation.[34] Furthermore, Fargeas *et al.*[35] reported that prominin-1 has 12 splice variants carrying the AC133 epitope, and therefore it is possible that CD133 may be expressed but not recognized by our antibody. In addition, further analysis will be needed to clarify the discrepancies between our results and the results from studies on cell lines.

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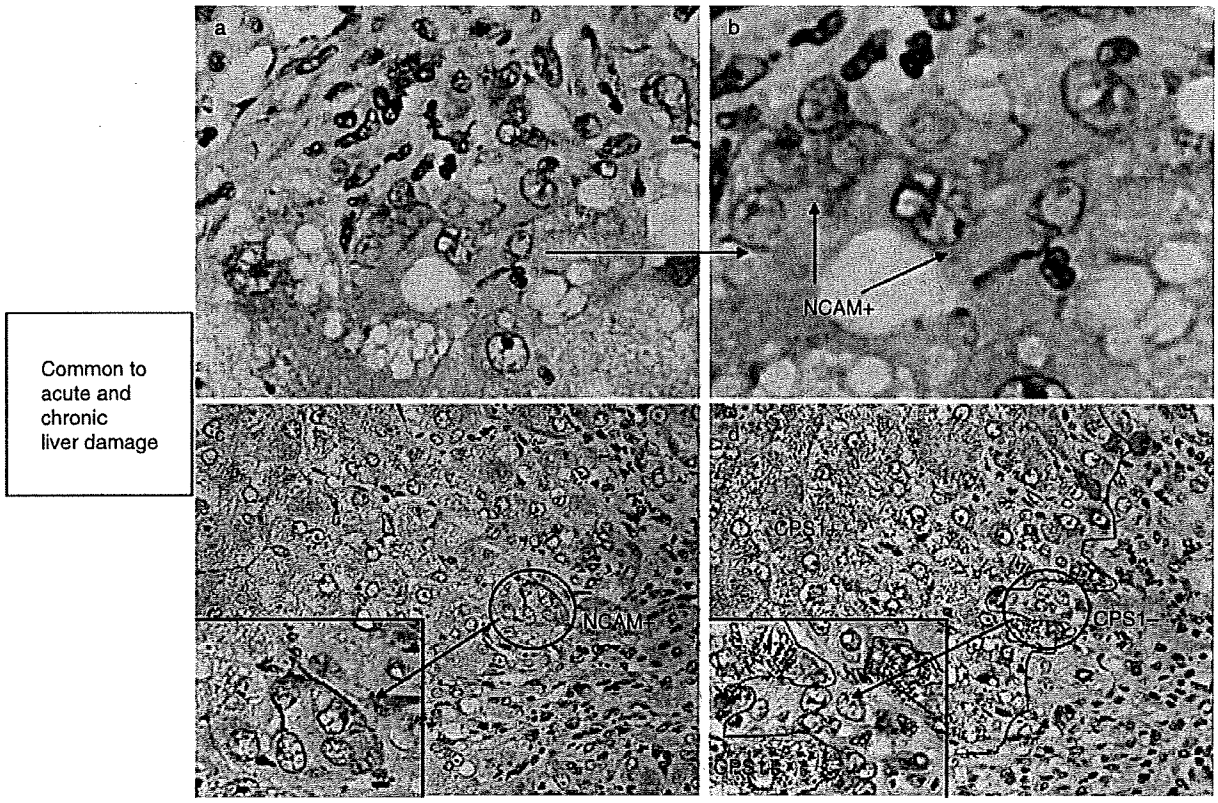

Figure 5 Analysis of the relationship between neural cell adhesion molecule (NCAM)$^+$ cells and mature hepatocytes. NCAM$^+$ cells that morphologically resembled hepatocytes could be detected in both acute and chronically damaged livers (a,b). Serial section analysis (c,d) revealed that NCAM$^+$ cells did not express the mature hepatocytic lineage marker carbomyl phosphate synthase (CPSI). Similar results were seen in other patients. Original magnifications (a) × 400; (c,d) × 200. (a,b) Case 11 in Table 1; (c,d) case 12 in Table 1.

The role of NCAM in the liver has not been reported. In the nervous system, the involvement of NCAM in growth, guidance and migration of neural crest cells and neurons, axon bundling, interaction of motor axons and muscle, and several other morphogenic events has been reported.[36,37] In the liver, NCAM$^+$ cells may also play these roles and, in our study, NCAM$^+$ cells could be detected in 9.7% of HCC. NCAM$^+$ cancers are not restricted to HCC and have also been reported in neuroblastoma, glioma, myeloma, thyroid cancer and small cell lung carcinomas.[29] In these cancers, NCAM can exert both positive and negative effects on cancer progression. In the field of gastroenterological cancer, Perl *et al.*[38] reported that reduced expression of NCAM induced metastatic dissemination of pancreatic β tumor cells, and Kameda *et al.*[39] reported that expression of NCAM was implicated in the induction of neural invasion in pancreatic cancers. Roesler *et al.*,[27] on the other hand, reported tumor suppressor activity of NCAM in colon adenocarcinoma. In HCC, neither well-differentiated nor poorly differentiated HCC expressed NCAM, although three cases with moderately differentiated HCC did express NCAM. Thus, it is unclear whether NCAM expression in HCC plays a positive or a negative role. Accumulation of NCAM$^+$ HCC cases and analysis of their clinical progression will help to determine whether NCAM$^+$ HCC would have a good or bad prognosis.

In this study, we have indicated that acute and chronically damaged livers contain large numbers of CD133$^+$ and NCAM$^+$ cells, and we have shown that DR can be identified using antibodies against CD133 and NCAM. Thus, it may be possible to use these two markers to enrich for CD133$^+$ and NCAM$^+$ hepatic stem/progenitor cells and to analyze these cells in more detail in order to clarify the regenerative system of the liver.

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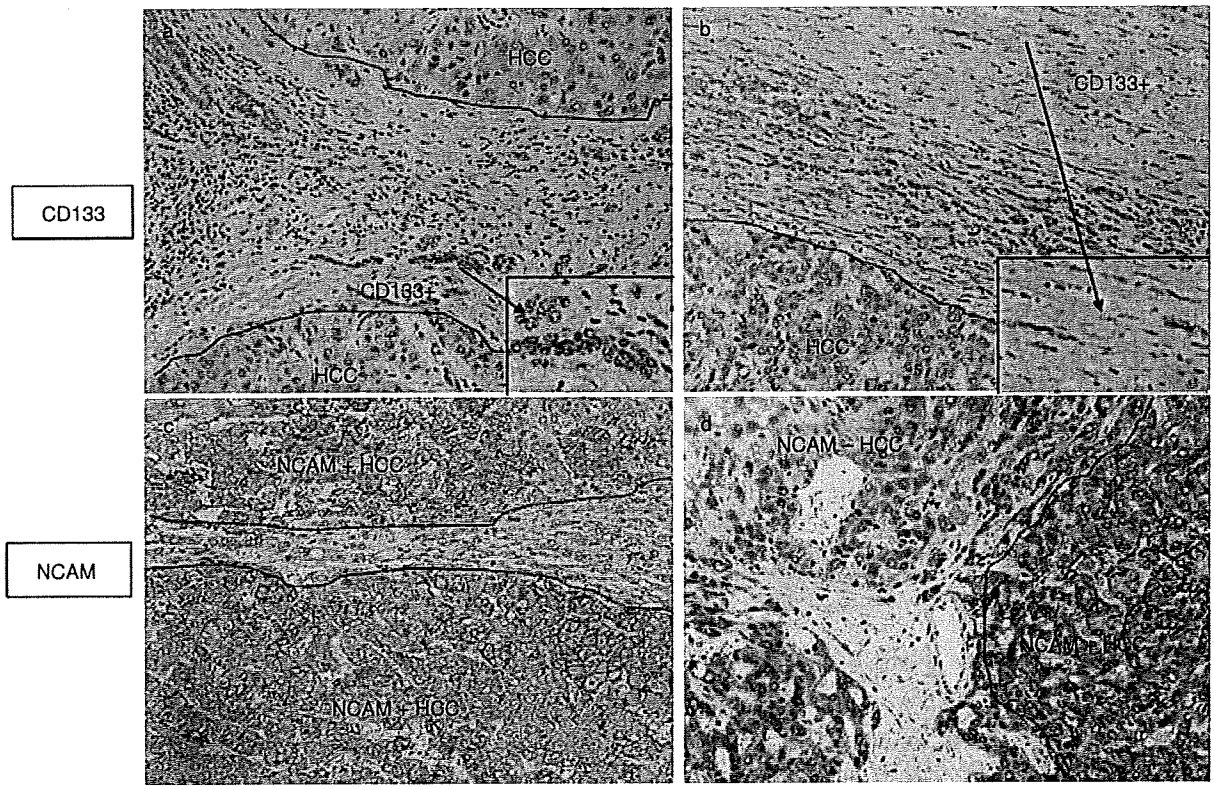

Figure 6 Analysis of CD133⁺ cells and neural cell adhesion molecule (NCAM)⁺ cells in hepatocellular carcinomas (HCC). CD133⁺ cells were not detected in HCC, while CD133⁺ ductular reactions (DR) could be detected in the fibrotic tissue present in (a) or around (b) the tumor. Case 1 (c) and case 5 (d) in Table 2 included a large number of NCAM⁺ cancer cells. Similar results were seen in other patients. Original magnification × 100. (a) Case 17 in Table 2; (b) case 18 in Table 2; (c) case 1 in Table 2; (d) case 5 in Table 2.

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ORIGINAL ARTICLE

Fucosylated Fraction of Alpha-Fetoprotein as a Predictor of Prognosis in Patients with Hepatocellular Carcinoma After Curative Treatment

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Abstract
Aim The aim of this study was to evaluate the clinical usefulness of measuring the *Lens culinaris* agglutinin-reactive fraction of alpha-fetoprotein (AFP-L3) for prognostic predictor in patients with hepatocellular carcinoma (HCC).
Methods A total of 477 HCC patients who underwent percutaneous ablative therapy or hepatectomy were enrolled. Overall survival and recurrence-free survival were respectively evaluated retrospectively and prospectively. Multivariate analyses of clinical prognostic factors were performed by Cox's stepwise proportional hazard model.
Results AFP-L3 status was a statistically significant independent prognostic factor of long-term survival ($P = 0.013$) and recurrence-free survival ($P = 0.006$) in patients who underwent percutaneous ablative therapy. In contrast, AFP-L3 did not affect prognosis in patients who underwent hepatectomy.
Conclusions AFP-L3 had different impacts on prognosis in patients with HCC who underwent percutaneous ablative therapy and hepatectomy. Our results suggest that AFP-L3 positivity (≥15%) might be a promising indicator for choosing therapeutic modalities in HCC patients.

Keywords Alpha-fetoprotein · AFP-L3 · DCP (des-γ-carboxy prothrombin) · Hepatocellular carcinoma · Prognostic factor

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Introduction

Hepatectomy is a generally accepted method that improves the long-term outcome in patients with hepatocellular carcinoma (HCC) [1]. However, patients with HCC frequently have coexisting liver cirrhosis with impaired hepatic functional reserve, and this may prevent surgical intervention. On the other hand, percutaneous ablative therapies, including percutaneous ethanol injection (PEI), microwave coagulation therapy (MCT), and percutaneous radiofrequency ablation (RFA), have been developed and applied as alternative therapeutic options in cases of small HCC [2–8]. Recently, RFA has been performed as a first-line therapeutic option for early stage HCC; its survival outcomes are similar to those of hepatectomy [6–8]. However, a method for making the correct choice among therapeutic modalities to suit individual patients with early stage HCC remains to be determined.

The *Lens culinaris* agglutinin-reactive fraction of alpha-fetoprotein (AFP-L3) has been reported to be a specific marker for HCC [9–11]. Moreover, its level predicts the

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malignant potential of HCC with subsequent unfavorable prognosis after treatment [12–16]. However, there have been few reports of the relationship between AFP-L3 status and prognosis in subgroups of HCC patients receiving different therapeutic modalities, such as hepatectomy and percutaneous ablative therapy.

The aim of this collaborative retrospective and prospective study was to evaluate the clinical usefulness of measuring AFP-L3 for prognostic predictor in patients with HCC after curative treatment.

Patients and Methods

Study Design

A total of 336 HCC patients underwent curative treatment at four participating hospitals (Niigata University Hospital, Ehime University Hospital, Shinsyu University Hospital, and Gunma University Hospital) from January 1998 to March 2005 and were investigated retrospectively. Of these patients, 232 underwent percutaneous ablative therapy and 104 underwent hepatectomy. Percutaneous ablative therapy comprised PEI in 90 patients, MCT in four patients, and RFA in 138 patients. Long-term survival data on these patients were confirmed as of the end of March 2005.

To evaluate the prognostic influence of AFP-L3 in two subgroups comparable for tumor extension, we prospectively investigated 189 patients diagnosed with early stage HCC initially at four hospitals from April 2005 to October 2007. We considered patients who had multiple (up to three) tumors measuring 3 cm or less in diameter as having early stage HCC. Forty-eight of 189 patients were excluded in this study, as they were received transcatheter treatment. As a result, 141 HCC patients, 99 who underwent percutaneous ablative therapy and 42 who underwent hepatectomy, were enrolled in the prospective study. Percutaneous ablative therapy comprised PEI in ten patients, MCT in two patients, and RFA in 87 patients. In these 141 patients, HCC recurrence was assessed by imaging modalities every 3 or 4 months after treatment and recurrence free survival was evaluated as of the end of December 2007. Informed consent was obtained from each patient, and the study protocol conformed with the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in the a priori approval by our institution's human research committee.

Diagnosis of HCC and Laboratory Examination

In our study, the diagnosis was based essentially on imaging findings together with increments of tumor marker levels. We employed methods such as computed tomography (CT), magnetic resonance imaging, and CT during hepatic arteriography, considering hyperattenuation in the arterial phase with washout in the late phase to be a typical feature of HCC. In nine cases that showed atypical features on imaging, ultrasound-guided biopsies were performed.

Hepatic functional reserve was ranked by the criteria of the Child-Pugh scoring system. Serum alpha-fetoprotein (AFP) and des-gamma-carboxy prothrombin (DCP) were determined at each hospital by using commercially available kits. AFP-L3 percentage was measured at each hospital by liquid-binding assay (Wako Pure Chemical Industries Ltd, Osaka, Japan) [17]. AFP, AFP-L3, and DCP were measured in the same serum before treatment. Cut-off values for positivity for AFP, AFP-L3, and DCP were set at 20 ng/ml, 15%, and 40 mAU/ml, respectively, based on previous studies [18–20].

Treatment

Therapeutic modalities for individual patients were chosen according to hepatic functional reserve, tumor multiplicity, and tumor size. Percutaneous local ablative therapies were performed under a US-guided procedure, and its efficacy was evaluated with dynamic CT within a few days after treatment. Complete ablation of HCC was defined as non-enhancement of the lesion with surrounding liver parenchyma. Patients received additional sessions of an ablative therapy until the treatment was judged as complete. During the study, a Cool-tip RF System attached to a 200-W power generator (Radionics, Burlington, Massachusetts, USA) was the main device used for RFA treatment and Microtaze OT-110M (Alfresa-Pharma Co., Inc., Osaka, Japan) was used for MCT.

Statistical Analysis

Differences in the proportions of the independent binary variables were determined by Fisher's exact test. Continuous variables were compared by Student's *t*-test. Univariate survival and recurrence-free survival were determined by the Kaplan–Meier method. Log-rank test was used to test for equality of long-term survival and recurrence-free survival between the groups. Multivariate analyses of prognostic factors in the clinical features were performed by using Cox's stepwise proportional hazard model. The factors included for multivariate analyses were patient age, gender (female/male), HBsAg (negative/positive), Anti-HCV (negative/positive), Child-Pugh class (A/B, C), AFP (ng/ml) ($<20/\geq 20$), DCP (mAU/ml) ($<40/\geq 40$), AFP-L3 (%) ($<15/\geq 15$), tumor size (cm) ($<3/\geq 3$ or $\leq 2/>2$), and number of tumors (single/multiple). Statistical analyses were performed with SPSS 15.0 software (SPSS Japan Inc. Tokyo, Japan). A *P*-value of less than 0.05 was considered as statistically significant.

Results

Retrospective Study

Clinical Features of Patients Classified by Therapeutic Modality

A total of 336 HCC patients who underwent hepatectomy and percutaneous ablative therapy were investigated retrospectively. Patients who underwent percutaneous ablative therapy were characterized by older age ($P < 0.05$), positivity for antibody to hepatitis C virus (anti-HCV) ($P < 0.05$), and advanced Child-Pugh classification ($P < 0.05$). In contrast, patients who underwent hepatectomy were characterized by positivity for hepatitis B surface antigen (HBsAg) ($P < 0.05$), AFP-L3 ($P < 0.05$), and DCP ($P < 0.05$) elevation, as well as large tumor size ($P < 0.05$). No significant differences were observed between the two groups in terms of gender, AFP level, or number of tumors (Table 1A).

Univariate and Multivariate Analyses of the Factors Predicting Long-Term Patient Survival

The median observation time after treatment was 38.3 months (range, 1.0–146.2 months). Of the 232 patients who underwent percutaneous ablative therapy, 172 were alive and 60 had died from HCC, hepatic failure, and/or complications of cirrhosis. Of the 104 HCC patients who underwent hepatectomy, 68 were alive and 36 had died. The median survival time was 69.0 months in patients who had undergone percutaneous ablative therapy and 114.9 months in those who had undergone hepatectomy.

In the univariate analysis, anti-HCV status ($P = 0.034$), AFP status ($P = 0.007$), AFP-L3 status ($P = 0.001$), tumor size ($P = 0.001$), and number of tumors ($P = 0.045$) were significant prognostic factors of long-term survival in patients who underwent percutaneous ablative therapy. AFP status ($P = 0.011$), tumor size ($P = 0.006$), and number of tumors ($P < 0.001$) were significant prognostic factors in patients who underwent hepatectomy (Table 2).

Multivariate analysis by Cox's stepwise proportional hazard model revealed that tumor size ($P = 0.018$) and AFP-L3 status ($P = 0.013$) were significant independent prognostic factors for long-term survival in patients who underwent percutaneous ablative therapy. Tumor size ($P = 0.013$) and number of tumors ($P = 0.004$) were significant independent prognostic factors in patients who underwent hepatectomy (Table 3). We showed the long-term survival curves of two groups (with or without AFP-L3 elevation) in patients who underwent percutaneous ablative therapy and in those who underwent hepatectomy (Fig. 1). No significant difference in survival was observed

Table 1 Clinical features of patients with HCC classified by therapeutic modality in the retrospective and prospective studies

Variables	Percutaneous ablation ($n = 232$)	Hepatectomy ($n = 104$)
(A) Retrospective study		
Age (median, range)	68 (39–89)	65 (35–81)*
Gender		
Male	145 (62.5%)	66 (63.5%)
Female	87 (37.5%)	38 (36.5%)
HBsAg		
Negative	209 (90.1%)	73 (70.2%)
Positive	23 (9.9%)	31 (29.8%)*
Anti-HCV		
Negative	28 (12.1%)	45 (43.3%)
Positive	204 (87.9%)	59 (56.7%)*
Child-Pugh class		
A	177 (76.3%)	95 (91.3%)
B and C	55 (23.7%)	9 (8.7%)*
AFP (ng/ml)		
<20	65 (28.0%)	22 (21.2%)
≥20	167 (72.0%)	82 (78.8%)
DCP (mAU/ml)		
<40	149 (67.4%)	48 (51.1%)
≥40	72 (32.6%)	46 (48.9%)*
AFP-L3 (%)		
<15	181 (78.0%)	61 (58.7%)
≥15	51 (22.0%)	43 (41.3%)*
Tumor size (cm)		
<3	185 (79.7%)	33 (31.7%)
≥3	47 (20.3%)	71 (68.3%)*
Tumor number		
Single	148 (63.8%)	75 (72.1%)
Multiple	84 (36.2%)	29 (27.9%)
Variables	Percutaneous ablation ($n = 99$)	Hepatectomy ($n = 42$)
(B) Prospective study		
Age (median, range)	69 (36–85)	65 (40–80)
Gender		
Male	66 (66.7%)	24 (57.1%)
Female	33 (33.3%)	18 (42.9%)
HBsAg		
Negative	85 (85.9%)	29 (69.0%)
Positive	14 (14.1%)	13 (31.0%)*
Anti-HCV		
Negative	27 (27.3%)	15 (35.7%)
Positive	72 (72.7%)	27 (64.3%)
Child-Pugh class		
A	79 (79.8%)	39 (92.9%)
B and C	20 (20.2%)	3 (7.1%)

Table 1 continued

Variables	Percutaneous ablation ($n = 99$)	Hepatectomy ($n = 42$)
AFP (ng/ml)		
<20	64 (64.6%)	22 (52.40%)
≥20	35 (35.4%)	20 (47.6%)
DCP (mAU/ml)		
<40	63 (63.6%)	27 (64.3%)
≥40	35 (35.4%)	15 (35.7%)
AFP-L3 (%)		
<15	85 (85.9%)	33 (78.6%)
≥15	14 (14.1%)	9 (21.4%)
Tumor size (cm)		
≤2	63 (63.6%)	27 (64.3%)
>2	36 (36.4%)	15 (35.7%)
Tumor number		
Single	78 (78.8%)	34 (81.0%)
Multiple	21 (21.2%)	8 (19.0%)

HBsAg hepatitis B surface antigen, *HCV* hepatitis C virus, *AFP* alpha-fetoprotein, *DCP* des-gamma-carboxy prothrombin. Percentages are shown in parentheses

* $P < 0.05$ between groups by Fisher's exact test and Student's *t*-test

between the two AFP-L3 groups in patients who underwent hepatectomy ($P = 0.308$). In contrast, patients in the ablative therapy group whose AFP-L3 levels were below 15% lived significantly longer than those whose values were more than 15% ($P = 0.001$).

Prospective Study

Clinical Features of Patients with Early Stage HCC Classified by Therapeutic Modality

A total of 141 patients with early stage HCC were evaluated prospectively. Patients who underwent hepatectomy were characterized by positive for hepatitis B surface antigen (HBsAg) ($P < 0.05$). No significant differences were observed in age, gender, anti-HCV positivity, AFP status, AFP-L3 status, DCP status tumor size, and number of tumors between the two groups. Patients who underwent percutaneous ablative therapies tended to have an advanced Child-Pugh classification ($P = 0.055$) (Table 1B).

Univariate and Multivariate Analysis of the Factors Predicting Recurrence-Free Survival in Patients with Early Stage HCC

The median follow-up time after treatment was 12.0 months (range, 1.0–30.5 months). Among the 99 patients who underwent percutaneous ablation, recurrences were observed in 36 (36.4%). Among the 42 patients who underwent hepatectomy, recurrences were observed in six (14.3%).

In the univariate analysis, we found no significant difference in recurrence-free survival rates by pretreatment variables in patients who underwent percutaneous ablation, although AFP-L3 elevation ($P = 0.054$) tended to decrease recurrence-free survival. In contrast, tumor size ($P = 0.038$) and number of tumors ($P = 0.034$) were significant prognostic factors in patients who underwent hepatectomy (Table 2).

Although this prospective study was conducted over a short period of time, multivariate analysis of prognostic factors among the clinical features was performed and Cox's stepwise proportional hazard model revealed that HBsAg status ($P = 0.033$), DCP status ($P = 0.011$), and AFP-L3 status ($P = 0.006$) were significant independent prognostic factors of recurrence-free survival in patients who underwent percutaneous ablative therapies. On the other hand, we found no significant independent prognostic factors in patients who underwent hepatectomy (Table 3).

We showed recurrence-free survival rates between two groups—with or without AFP-L3 elevation—among

Table 2 Univariate analysis of the factors predicting long-term survival in the retrospective study and recurrence-free survival in the prospective study for patients who underwent percutaneous ablation and in those who underwent hepatectomy

Variables	Long-term survival		Recurrence-free survival	
	Percutaneous ablation *P*-value	Hepatectomy *P*-value	Percutaneous ablation *P*-value	Hepatectomy *P*-value
Gender (female/male)	0.907	0.525	0.225	0.194
HBsAg (negative/positive)	0.139	0.801	0.151	0.314
Anti-HCV (negative/positive)	0.034	0.963	0.194	0.171
Child-Pugh class (A/B,C)	0.083	0.235	0.293	0.487
AFP (ng/ml) (<20/≥20)	0.007	0.011	0.117	0.994
DCP (mAU/ml) (<40/≥40)	0.328	0.153	0.075	0.059
AFP-L3 (%) (<15/≥15)	0.001	0.308	0.054	0.530
Tumor size (cm) (<3/≥3)	0.001	0.006	0.063	0.038
Tumor number (single/multiple)	0.045	<0.001	0.667	0.034

HBsAg hepatitis B surface antigen, *HCV* hepatitis C virus, *AFP* alpha-fetoprotein, *DCP* des-gamma-carboxy prothrombin. *P*-value was calculated using Log-rank test

Table 3 Multivariate analysis of factors predicting long-term survival in the retrospective study and recurrence-free survival in the prospective study for patients who underwent percutaneous ablation and in those who underwent hepatectomy

Long-term survival			Recurrence-free survival		
Variables	Hazard ratio (95% CI)	*P*-value	Variables	Hazard ratio (95% CI)	*P*-value
Percutaneous ablation			Percutaneous ablation		
AFP-L3 (%)			HBsAg		
<15	1		Negative	1	
≥15	2.098 (1.169–3.765)	0.013	Positive	2.823 (1.090–7.310)	0.033
Tumor size (cm)			DCP		
<3	1		<40 (mAU/ml)	1	
≥3	1.998 (1.123–3.553)	0.018	≥40 (mAU/ml)	2.767 (1.267–6.046)	0.011
			AFP-L3		
			<15 (%)	1	
			≥15 (%)	3.463 (1.437–8.347)	0.006
Hepatectomy			Hepatectomy		
Tumor size (cm)			Tumor number		
<3	1		Single	1	
≥3	6.162 (1.457–26.064)	0.013	Multiple	4.654 (0.936–23.149)	0.060
Tumor number					
Single	1				
Multiple	3.170 (1.442–6.921)	0.004			

Hazard ratio and *P*-value were calculated using Cox's stepwise proportional hazard model

CI confidence interval, *AFP* alpha-fetoprotein, *HBsAg* hepatitis B surface antigen, *DCP* des-gamma-carboxy prothrombin

patients with early stage HCC who underwent percutaneous ablation and patients who underwent hepatectomy (Fig. 1). No significant difference was observed between groups with or without AFP-L3 elevation ($P = 0.53$) in patients who underwent hepatectomy. In contrast, a close-to-significant ($P = 0.054$) difference was observed between the groups of patients with and without AFP-L3 elevation who underwent percutaneous ablative therapy.

In summary, the results of the retrospective and prospective studies demonstrated that AFP-L3 status was a statistically significant prognostic factor of long-term survival and recurrence-free survival in patients who underwent percutaneous ablative therapy, but did not affect prognosis in patients who underwent hepatectomy.

Discussion

AFP-L3, a fucosylated species of AFP, is the product of alpha 1-6 fucosyltransferase (FUT8) in the presence of GDP-fucose. Our previous result revealed that FUT8 levels in HCC tissue were higher than those in the surrounding non-cancerous tissues and that FUT8 levels of HCC tissue increased in accordance with tumor dedifferentiation [21]. Several reports have shown the relationship between AFP-L3 status and histologic grade in HCC. Miyaaki et al. [16] showed that the frequency of poorly differentiated HCC was significantly higher in AFP-L3-positive patients than in AFP-L3-negative patients. Oka et al. [14] reported that AFP-L3-positive HCC was characterized by portal vein invasion and poorer differentiation, and that tumors in AFP-L3-positive HCC were advanced, even if they were small and the patient had a low serum AFP concentration. These results indicate the relationship between increased AFP-L3 level and increased degree of malignant behavior of HCC tissue.

Recurrence after treatment is an important factor affecting prognosis. Vascular invasion is an established adverse prognostic indicator of recurrence of HCC [22, 23]. Yamashita et al. [24] suggested that portal vein invasion is associated with AFP-L3 positivity, and that there is a strong possibility of intrahepatic invasion when there is positive conversion of this marker. Hayashi et al. [13] reported the relationship between AFP-L3 status and pattern of recurrence in patients with HCC. In their report, intrahepatic metastasis was significantly more common in AFP-L3-positive patients than in negative patients, although the recurrence rate of multicentric tumors did not differ significantly between the two groups with or without AFP-L3 elevation. From this point of view, hepatectomy—especially anatomical resection, which can remove venous tumor thrombi together with the primary lesion—is more suitable than local ablative therapies for the treatment of AFP-L3-positive patients.

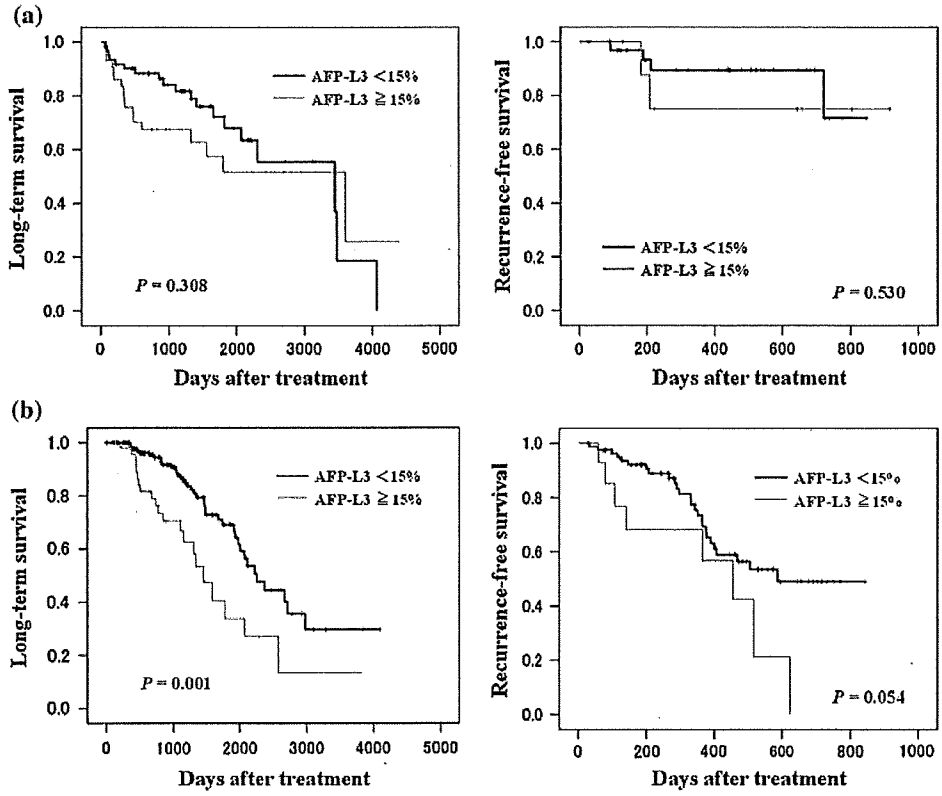

Fig. 1 Comparison of long-term survival rates and recurrence-free survival rates between patients with and without AFP-L3 elevation who underwent hepatectomy (a) and who underwent percutaneous ablation (b)

In our study, the pathological diagnosis was made by individual pathologists at each hospital. At Niigata University Hospital, 58 HCC patients underwent hepatectomy, of whom 23 had an elevated serum AFP-L3 level (≥15%) and the remaining 35 were negative for AFP-L3 (<15%). Among the 23 patients with AFP-L3 elevation, only two (8.7%) were diagnosed as having well-differentiated HCC on the basis of the resected specimens, 14 (60.9%) had moderately differentiated HCC, and seven (30.4%) had poorly differentiated HCC. In contrast, among the 35 patients who were negative for AFP-L3, 7 (20.0%) were diagnosed as having well-differentiated HCC, 24 (68.6%) had moderately differentiated HCC, and only four (11.4%) had poorly differentiated HCC. Although no statistically significant differences were observed by Fisher's exact test, the group showing AFP-L3 elevation tended to have a poorer histopathological grading ($P = 0.141$). Only eight out of 331 patients who underwent percutaneous ablative therapy were diagnosed as having HCC on the basis of histological findings in four hospitals. Therefore, we were unable to investigate whether poorly differentiated tumors were more frequent in the groups who underwent percutaneous ablative therapy and hepatectomy. Portal vein invasion was investigated similarly in 58 patients, and was found to be present in six of 23 AFP-L3-positive patients and six of 35 AFP-L3-negative patients. No significant difference was observed between AFP-L3 and portal vein invasion in this limited investigation.

We demonstrated here in a multicenter retrospective study that AFP-L3 status was a significant prognostic factor affecting the long-term survival of patients who underwent percutaneous ablative therapy. In addition, to evaluate the prognostic influence of AFP-L3 in two subgroups comparable for tumor extension, we performed a multicenter prospective study to identify the prognostic factors for recurrence-free survival in patients with early stage HCC. Although this evaluation was conducted over a short period of time, we confirmed that AFP-L3 status was a significant prognostic predictor of recurrence-free survival in patients who underwent percutaneous ablative therapy, but it did not affect the prognosis of patients who underwent hepatectomy.

A number of studies have shown that AFP-L3 status is an independent prognostic factor in patients with HCC [12, 13, 15]. We previously reported that AFP-L3-positive (>15%) patients had a lower survival rate than negative (<15%) patients in subgroups with a low serum AFP concentration. Moreover, the statistically significant differences were more distinct in the subgroups with lower AFP concentrations [20]. However, the patients in these studies had received various treatments such as hepatectomy, RFA, and transcatheter arterial embolization, and

there have been few reports of the relationship between AFP-L3 status and prognosis in subgroups of HCC patients receiving different therapeutic modalities. Tateishi et al. [15] demonstrated that pre-treatment AFP-L3 positivity (>15%) was a significant predictor of HCC recurrence in patients who underwent curative ablation, and that AFP-L3 positivity after ablation was the strongest predictor of HCC recurrence by multivariate analysis. Although their study was performed in only one center and did not evaluate long-term survival, their results are compatible with ours.

Treatment of HCC patients with cirrhosis faces a dilemma in that minimization of damage to noncancerous liver tissue improves long-term survival, but incomplete treatment of subsequent HCC recurrences results in a poor prognosis. Accordingly, if a useful indicator of choice of therapeutic modality were to be available before the initial therapy, there would be several advantages in not only the treatment, but also the follow-up, of patients with HCC.

In conclusion, present results revealed that AFP-L3 had different impacts on prognosis in patients with HCC who underwent percutaneous ablative therapy and hepatectomy. Although this study was not a randomized control trial, AFP-L3 might be a promising scale to improve the prognostic estimate and appraisal of therapeutic outcome in patients with HCC.

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ORIGINAL ARTICLE

Characterization of CD133$^+$ parenchymal cells in the liver: Histology and culture

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Abstract

AIM: To reveal the characteristics of CD133$^+$ cells in the liver.

METHODS: This study examined the histological characteristics of CD133$^+$ cells in non-neoplastic and neoplastic liver tissues by immunostaining, and also analyzed the biological characteristics of CD133$^+$ cells derived from human hepatocellular carcinoma (HCC) or cholangiocarcinoma cell lines.

RESULTS: Immunostaining revealed constant expression of CD133 in non-neoplastic and neoplastic biliary epithelium, and these cells had the immunophenotype CD133$^+$/CK19$^+$/HepPar-1$^-$. A small number of CD133$^+$/CK19$^-$/HepPar-1$^+$ cells were also identified in HCC and combined hepatocellular and cholangiocarcinoma. In addition, small ductal structures, resembling the canal of Hering, partly surrounded by hepatocytes were positive for CD133. CD133 expression was observed in three HCC (HuH7, PLC5 and HepG2) and two cholangiocarcinoma cell lines (HuCCT1 and CCKS1). Fluorescence-activated cell sorting (FACS) revealed that CD133$^+$ and CD133$^-$ cells derived from HuH7 and HuCCT1 cells similarly produced CD133$^+$ and CD133$^-$ cells during subculture. To examine the relationship between CD133$^+$ cells and the side population (SP) phenotype, FACS was performed using Hoechst 33342 and a monoclonal antibody against CD133. The ratios of CD133$^+$/CD133$^-$ cells were almost identical in the SP and non-SP in HuH7. In addition, four different cellular populations (SP/CD133$^+$, SP/CD133$^-$, non-SP/CD133$^+$, and non-SP/CD133$^-$) could similarly produce CD133$^+$ and CD133$^-$ cells during subculture.

CONCLUSION: This study revealed that CD133 could be a biliary and progenitor cell marker *in vivo*. However, CD133 alone is not sufficient to detect tumor-initiating cells in cell lines.

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Key words: Cholangiocarcinoma; Hepatocellular carcinoma; Keratins; Stem cells

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INTRODUCTION

CD133 (also known as prominin-1 or AC133) was the first identified member of the prominin family of pentaspan membrane proteins[1-3]. In 1997, CD133 was reported as a marker of hematopoietic progenitor cells, using a novel monoclonal antibody that recognized the CD133 antigen[1-3]. Subsequently, it was reported that CD133 was also expressed in epithelial and non-epithelial progenitor cells in murine or human tissues including brain, kidney, prostate, pancreas, and skin[4-8].

The specific functions and ligands of CD133 have not been elucidated completely, although CD133 currently is recognized as a stem cell marker for normal and cancerous tissues in various organs[9-13].

Until now, there have been several reports regarding CD133 expression in hepatocellular carcinoma (HCC)[14-17]. Suetsugu *et al*[14] have examined CD133 expression in three cell lines of human HCC (HuH7, HepG2 and Hc). CD133 is expressed only on the surface of HuH7 cells. The CD133$^+$ population of HuH7 cells is characterized by high proliferation activity and lower expression of mature hepatocellular markers. CD133$^+$ cells can form tumors in SCID mice, whereas CD133$^-$ cells induce a very small number of tumors or none at all. It has been concluded that CD133 could be useable as a marker of cancer stem cells in human HCC[14]. Yin *et al*[15] and Ma *et al*[16] also have characterized CD133$^+$ cells in HCC, and they have reached a conclusion similar to that of Suetsugu *et al*[14]. However, because these previous studies were mainly *in vitro*, the histological characteristics of hepatic CD133$^+$ cells have not been fully examined so far. In particular, there are few data about CD133$^+$ cells in non-neoplastic liver tissues and non-hepatocellular liver cancers.

In this study, CD133 expression in non-neoplastic and neoplastic liver tissues was examined. *In vitro* studies were also performed to examine the biological characteristics of CD133$^+$ cells of HCC and cholangiocarcinoma cell lines. The goal of this study was to elucidate the histological and biological characteristics of CD133$^+$ cells in non-neoplastic and neoplastic human livers.

MATERIALS AND METHODS

Histological studies

Case selection: A total of 52 samples of liver tissues were obtained from the hepatobiliary disease file of the Division of Pathology, Kanazawa University Hospital in Japan between 2005 and 2009. This study consisted of three cases of normal liver, five cases of chronic viral hepatitis or liver cirrhosis, 33 cases of HCC, six cases of intrahepatic cholangiocarcinoma, and five cases of combined hepatocellular and cholangiocarcinoma (combined carcinoma). All cases used in this study were surgically resected cases. Normal liver tissues used in this study were background liver tissues of metastatic colon cancers. Age, sex and clinicopathological characteristics are shown in Table 1.

Expression of CD133 (mRNA level): Total RNA was extracted from the frozen section of all 47 cases using an RNeasy Mini kit (Qiagen, Valencia, CA, USA). Total RNA was dissolved in 50 μL of distilled water that contained 0.1% diethylpyrocarbonate, and quantitated using a spectrophotometer at OD_{260}. Isolated RNA was used for the subsequent reverse transcriptase-polymerase chain reaction (RT-PCR). The expression of CD133 mRNA was examined by nested RT-PCR using two sets of primers. The oligonucleotide sequences, numbers of cycles, and annealing temperatures of these primers are shown in Table 2. After PCR, 5-μL aliquots of the products were subjected to 1.5% or 2.0% agarose gel electrophoresis and stained with ethidium bromide.

Immunostaining of CD133, cytokeratin 19 (CK19) and hepatocyte paraffin-1 (HepPar-1): Frozen sections of 52 samples of non-neoplastic and neoplastic liver tissues were used for immunostaining. Immunostaining for CD133, CK19 and HepPar-1 was performed using a mouse monoclonal antibody against human CD133 (clone AC133; Miltenyi Biotec, Auburn, CA, USA), a mouse monoclonal antibody against human CK19 (Dako Cytomation, Glostrup, Denmark), and a mouse monoclonal antibody against human HepPar-1 (Dako Cytomation).

Serial sections were used in each case to examine the co-localization of CD133, CK19 and HepPar-1 expression. Sliced frozen sections were fixed with acetone for 20 min. After blocking endogenous peroxidases, the sections were incubated in protein block solution (Dako Cytomation) for 20 min and incubated at 4℃ with each primary antibody. These sections were incubated for 1 h at room temperature with goat anti-mouse immunoglobulins, which were conjugated to peroxidase-labeled polymer (Envision+; Dako Cytomation). 3,3'-Diaminobenzidine tetrahydrochloride was used as the chromogen, followed by light counterstaining with hematoxylin. Negative controls were evaluated by substituting the primary antibody with similarly diluted non-immunized mouse serum.

Culture studies

Cell culture: Three human HCC cell lines (HuH7, PLC5 and HepG2) and two human cholangiocarcinoma cell lines (CCKS1 and HuCCT1) were used in this study. HuH7, PLC5 and HepG2 were obtained from the Hearth Science Research Bank (Osaka, Japan). HuCCT-1 was obtained from the Cell Resource Center for Biochemical Research, Tohoku University, Sendai, Japan. CCKS1 was established in our laboratory[18]. HuH7 and PLC5 were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen Corp., Carlsbad, CA, USA), and HepG2 was maintained in minimum essential medium (Invitrogen Corp.) with 1% nonessential amino acids (Specialty Media, Phillipsburg, NJ, USA). CCKS1 and HuCCT1 were cultured in RPMI-1640 medium (Invitrogen Corp.) Each medium was supplemented with 10% fetal bovine serum (Invitrogen Corp.) and 1% antibiotic-antimycotic (Invitrogen Corp.).

Dual fluorescent immunostaining of CD133/CK19 and CD133/alpha-fetoprotein (AFP): Cell lines were cultured on Lab-Tek II chamber slides (Nalge Nunc International, Naperville, IL, USA) for fluorescent immunostaining. After culturing for 2 d, the specimens were fixed in 4% paraformaldehyde for 10 min at 4℃. After incubation in protein block solution (Dako Cytomation) for 10 min, the specimens were incubated

Table 1 Age, sex, and etiology of liver diseases in our study

	n	Age (yr)	Male/Female	Etiology
Normal liver	3	50	2/1	
Chronic hepatitis/cirrhosis	5	58	3/2	HBV (3), HCV (2)
HCC				
Well-differentiated	3	62	2/1	HBV (1), HCV (2)
Moderately differentiated	24	62	20/4	HBV (11), HCV (7), alcohol (3), NASH (1), cryptogenic (1)[1]
Poorly differentiated	6	54	4/2	HBV (3)
Cholangiocarcinoma	6	60	3/3	HCV (4)
Combined carcinoma	5	59	4/1	HBV (1), HCV (4)

[1]The remaining one case had no etiology of liver diseases and showed histologically normal liver. NASH: Nonalcoholic steatohepatitis; HCC: Human hepatocellular carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

Table 2 Sequences, annealing temperatures, cycle times, and product sizes of PCR primers

	F/R	Sequence	Temperature (℃)	Cycles	Size (bp)
CD133[1]	1st F	GCCAGAAACTGTAATCTTAG	48	35	275
	1st R	TTACCTGGTGATTTGCCACA			
	2nd F	CCTGGGGCTGCTGTTTATTA	55	35	153
	2nd R	ATCACCAACAGGGAGATTGC			
CK19	F	TCCCGCGACTACAGCCACTACTACACGACC	55	35	745
	R	CGCGACTTGATGTCCATGAGCCGCTGGTA			
CK7	F	GGATGCTGCCTACATGAGC	52	30	164
	R	CCAGGGAGCGACTGTTGT			
AFP	F	GGGAGCGGCTGACATTATTA	50	30	231
	R	TCTTGCTTCATCGTTTGCAG			
Albumin	F	TGCTTGAATGTGCTGATGACAGGG	50	30	161
	R	AAGGCAAGTCAGCAGGCATCTCATC			
β-actin	F	CAAGAGATGGCCACGGCTGCT	55	30	334
	R	TCCTTCTGCATCCTGTCGGCA			

[1]Examined by nested PCR using first and second sets of primers. F: Forward; R: Reverse.

with antibodies against CD133 and CK19, or antibodies against CD133 and AFP for 1 h at room temperature. The antibodies used were as follows: CD133, mouse monoclonal, clone AC133, Miltenyi Biotec; CK19, goat polyclonal, clone G-14, Santa Cruz Biotechnology (Santa Cruz, CA, USA); and AFP, a rabbit polyclonal, Dako Cytomation. The reaction product was visualized with fluorescent goat anti-mouse and anti-rabbit IgG antibodies (1:500, Molecular Probes Inc., Eugene, OR, USA). Specimens were counterstained with DAPI (Molecular Probes Inc.), and fluorescent signals were observed using a fluorescence microscope (Olympus, Tokyo, Japan).

Fluorescence-activated cell sorting (FACS) with reference to CD133 expression: HuH7 and HuCCT1 cells were used for FACS. Cultured cells were harvested after treatment with 0.25% of trypsin-EDTA solution (Sigma Chemical Co., St Louis, MO, USA) for 20 min, and washed three times in Hanks' Balanced Salt Solution (Invitrogen Corp.). Cultured cells were stained live in a staining solution containing bovine serum albumin, insulin, and phycoerythrin (PE)-conjugated monoclonal antibody to CD133 (clone AC133; Miltenyi Biotec) for 30 min at 4℃. As negative controls, cultured cells were incubated similarly with non-immunized mouse immunoglobulin. Samples were analyzed and sorted by JSAN (Bay Bioscience, Kobe, Japan). Cell debris and cell aggregates were gated out electronically. For the positive population, only the top 5%-10% of the most brightly stained cells were selected. For the negative population, only the bottom 5%-10% of the most dimly stained cells were selected. Then, 1.0×10^5 cells were sorted from the positive or negative population at the most specific mode. Sorted cells were plated on culture dishes for subculture. After sorting, $CD133^+$ and $CD133^-$ cells were cultured separately. After 4-wk culture, cultured cells were sorted again into $CD133^+$ and $CD133^-$ cells using flow cytometry to evaluate how the $CD133^+$ cell ratios were altered in each subpopulation. After subculturing for 3 or 4 wk, cultured cells were sorted again into $CD133^+$ and $CD133^-$ cells to evaluate how the $CD133^+$ or $CD133^-$ populations changed during subculture. The percentages of $CD133^+$ cells were calculated in a total of 1000-5000 cells in each group.

RNA expression in culture cells: Total RNA was extracted from five types of cultured cells using an RNeasy Mini Kit (Qiagen). Total RNA was similarly extracted from $CD133^+$ and $CD133^-$ cells. RT-PCR was performed for CD133, hepatocyte makers (AFP and albumin), biliary markers (CK19 and CK7 and β-actin.

The oligonucleotide sequences, numbers of cycles and annealing temperatures of these primers are shown in Table 2. After PCR, 5-μL aliquots of the products were subjected to 1.5% or 2.0% agarose gel electrophoresis and stained with ethidium bromide.

Real-time RT-PCR: The alterations of CD133 expression levels were examined in non-sorted or sorted (CD133$^+$ or CD133$^-$) cultured cells time-dependently (days 0, 7, 14, 21 and 28) after the passage or sorting. Real-time analysis was performed using premade CD133 and β-actin-specific primers and probes with the ABI Prism 7700 sequence detection system (PE Applied Biosystems, Warrington, UK). RT-PCR was done with the TaqMan Universal PCR Master Mix (PE Applied Biosystems) using 2 μL cDNA in a 25-μL final reaction mixture. Cycling conditions were as follows: incubation at 50℃ for 2 min, 10 min at 95℃, and 50 cycles of 15 s at 95℃ and 1 min at 60℃. CD133 was normalized (ΔCt) to β-actin from the Ct value of CD133. Each experiment was performed in triplicate, and the mean adopted.

Cell proliferation assay of CD133$^+$ and CD133$^-$ cells: CD133$^+$ and CD133$^-$ cells were plated on a Lab-Tek II chamber slide (Nalge Nunc International), and cultured for 7 d before the cell proliferation assay. Cell proliferation was assayed using BrdU. Cultured cells were incubated on slides with BrdU solution (10 mmol/L) at 37℃ for 30 min. After fixing with 70% ethanol (50 mmol/L glycine buffer solution, pH 2.0) for more than 20 min, the slides were incubated with anti-BrdU solution at 37℃ for 30 min. After additional incubation with IgG fluorochrome solution for 30 min, positive signals were detected by a fluorescence microscope (Olympus).

Relationship between side population (SP) and CD133$^+$ cells: SP is currently estimated as one of the most reliable stem cell phenotypes[19,20]. The relationship between SP and CD133$^+$ cells was examined by FACS. After detaching and washing, the cultured cells were then incubated at 37℃ for 90 min with 20 μg/mL Hoechst 33342 (Sigma Chemical Co.), PE-conjugated monoclonal antibody to CD133 (clone AC133; Miltenyi Biotec), bovine serum albumin, in the presence or absence of 100 μmol/L verapamil (Sigma Chemical Co.). After incubation, 1 μg/mL propidium iodide (Sigma Chemical Co.) was added and the cells were filtered through a 40-μm cell strainer (BD Biosciences, San Diego, CA, USA) to obtain single-cell suspensions. The relationship between SP and CD133 expression was analyzed by JSAN (Bay Bioscience). Hoechst 33342 was excited with a UV laser at 350 nm and fluorescence emission was measured with 405/BP30 (Hoechst blue) and 570/BP20 (Hoechst red) optical filters. Propidium iodide labeling was measured through a 630/BP30 filter for the discrimination of dead cells. Next, HuH7 cells were sorted into SP/CD133$^+$, SP/CD133$^-$, non-SP/CD133$^+$, and non-SP/CD133$^-$. After 4 wk subculturing, each population was analyzed again with regard to CD133 expression by FACS.

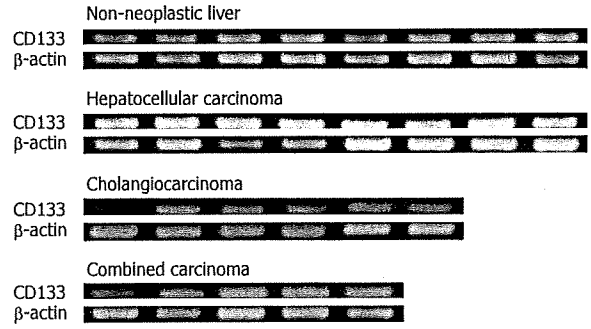

Figure 1 Expression of CD133 mRNA. Nested RT-PCR revealed CD133 mRNA expression in all cases of non-neoplastic liver tissue, HCC, intrahepatic cholangiocarcinoma, and combined hepatocellular and cholangiocarcinoma. Only eight cases of HCC are shown, although the remaining cases also expressed CD133 mRNA.

Statistical analysis

Differences between two groups were analyzed using the Mann-Whitney *U* test or χ^2 test. Statistical analysis was performed using Statcel 2 software (OMS publishing, Tokorozawa, Japan). $P < 0.05$ was considered to be significant.

RESULTS

CD133 expression in non-neoplastic and neoplastic liver tissues

The expression of CD133 mRNA was identified in all non-neoplastic and neoplastic liver tissues examined in this study by nested RT-PCR (Figure 1). The results of immunostaining of CD133 are shown in Figures 2 and 3. In normal livers, CD133 was expressed constantly in biliary epithelium of intrahepatic large and small bile ducts. Mature hepatocytes were negative for CD133. In the livers of chronic hepatitis and liver cirrhosis patients, CD133 was expressed in bile ducts and proliferating bile ductules. In addition, small ductal structures, resembling the canal of Hering, partly surrounded by hepatocytes were also positive for CD133 (Figure 2). CD133 was expressed on cellular membrane with accentuation on the luminal side. Immunostaining of CK19 and HepPar-1 on serial sections revealed that CD133 and CK19 expressions were closely co-localized (Figure 2). In contrast, mature hepatocytes that expressed HepPar-1 were negative for CD133. CD133 expression was not evident in mesenchymal or inflammatory cells upon immunostaining.

In HCC, eight of 33 cases (24%) had CD133$^+$ cells. CD133$^+$ cells were small in number and randomly distributed in these tumors. There were no morphological differences between CD133$^+$ and CD133$^-$ cells. Serial sections stained with CK19 and HepPar-1 revealed that CD133$^+$ cells in HCC were HepPar-1$^+$ and CK19$^-$ (Figure 3). CD133$^+$ cells were observed more often in

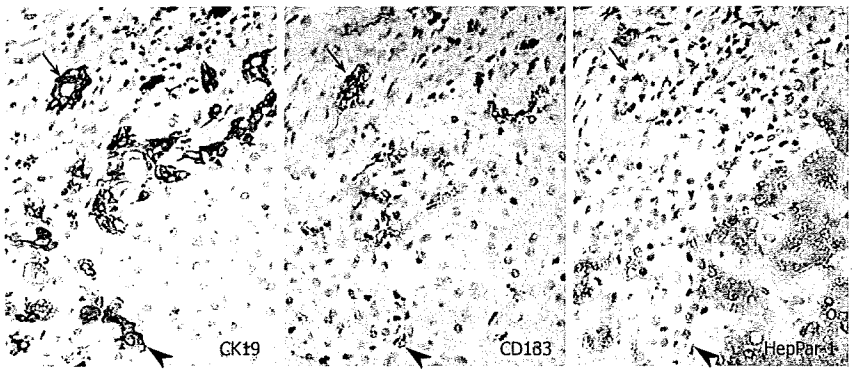

Figure 2 CD133 expression in liver cirrhosis (immunostaining). CD133 was expressed in bile duct (black arrows), bile ductules (white arrows), and small parenchymal cells surrounded by hepatocytes. CD133 was expressed on the cellular membrane with an accentuation on the luminal side. $CD133^+$ cells were also positive for CK19 but not HepPar-1. All images, × 400.

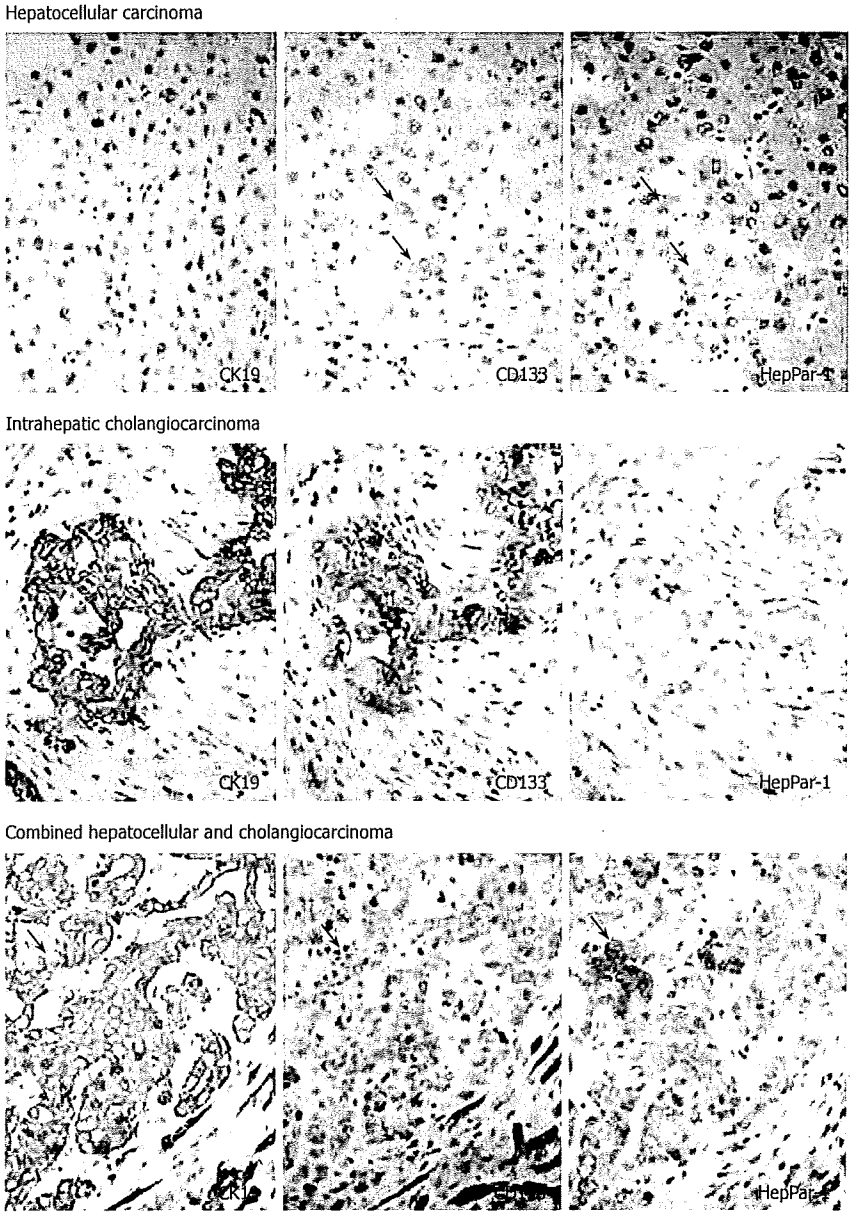

Figure 3 CD133 expression in HCC, intrahepatic cholangiocarcinoma, and combined hepatocellular and cholangiocarcinoma (immunostaining). In HCC, a few carcinoma cells expressed CD133, and those cells were $CK19^-$ and $HepPar\text{-}1^+$ (arrows). In cholangiocarcinoma, CD133 was expressed diffusely in carcinoma cells, and CK19 was also positive. In combined carcinoma, CD133 was expressed mainly in carcinoma cells positive for CK19, whereas some carcinoma cells were $CD133^+/CK19^-/HepPar\text{-}1^+$ (arrows). All images, × 400.

less differentiated HCCs: 0/3 (0%) in well-differentiated, 4/24 (17%) in moderately differentiated, and 4/6 (67%) in poorly differentiated HCC cases. The expression of CD133 mRNA was detected in all HCC cases by nested RT-PCR, although $CD133^+$ cells were identified in only 24% of cases by immunostaining. This discrepancy might have resulted from the small numbers of $CD133^+$ cells in HCC.

In cholangiocarcinoma, CD133 was expressed diffusely in carcinoma cells in all cases examined (Figure 3).

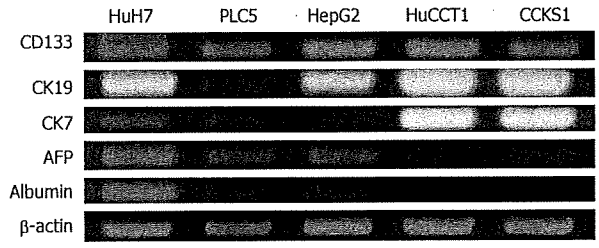

Figure 4 Expression levels of mRNA in cell lines. CD133 mRNA was expressed in all cell lines examined. Biliary markers (CK19 and CK7) were expressed more often in cholangiocarcinoma cell lines (HuCCT1 and CCKS1), whereas hepatocellular markers (AFP and albumin) were expressed constantly in HCC cell lines (HuH7, PLC5, and HepG2).

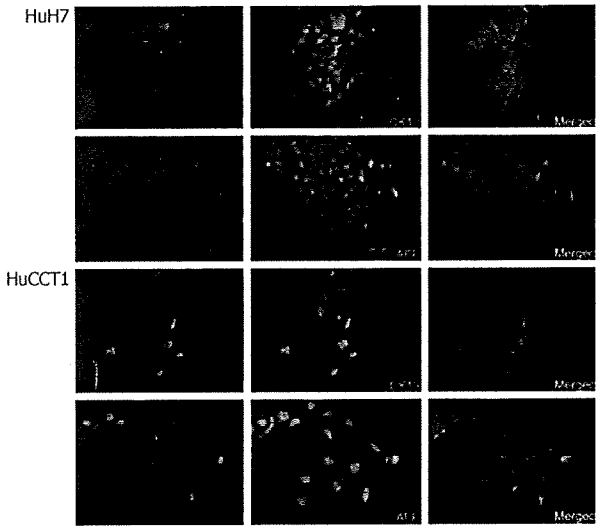

Figure 5 Dual immunofluorescence of CD133/CK19 and CD133/AFP in HuH7 and HuCCT1 cells. CD133$^+$ carcinoma cells were positive for CK19 or AFP in both HuH7 and HuCCT1 cell lines. All images, × 400.

CD133 expression was mainly on cellular membranes. HepPar-1 expression was not observed in any cases of cholangiocarcinoma, and CD133$^+$ cholangiocarcinoma cells were CK19$^+$ and HepPar-1$^-$. In combined carcinoma, all cases had CD133$^+$ carcinoma cells. CD133 expression was observed mainly in adenocarcinoma components. Most CD133$^+$ cells were CK19$^+$ and HepPar-1$^-$, although some CD133$^+$ cells were CK19$^-$ and HepPar-1$^+$ (Figure 3).

CD133 expression in cultured cells

The expression of CD133 mRNA was identified in all cell lines by RT-PCR (Figure 4). Biliary markers (CK19 and CK7) were expressed strongly in CCKS1 and HuCCT1 cells, whereas hepatocellular markers (AFP and albumin) were expressed constantly in HuH7, PLC5 and HepG2 cells. In addition, a cholangiocarcinoma cell line, HuCCT1, also expressed AFP. Similarly, HCC cell lines also expressed CK19 or CK7. Next, the relationships between CD133 and CK19 or AFP expression levels were examined using HCC (HuH7) and cholangiocarcinoma (HuCCT1) cell lines, both of which expressed hepatocellular and biliary markers. Dual immunostaining of CD133/CK19 or CD133/AFP revealed that CD133$^+$/

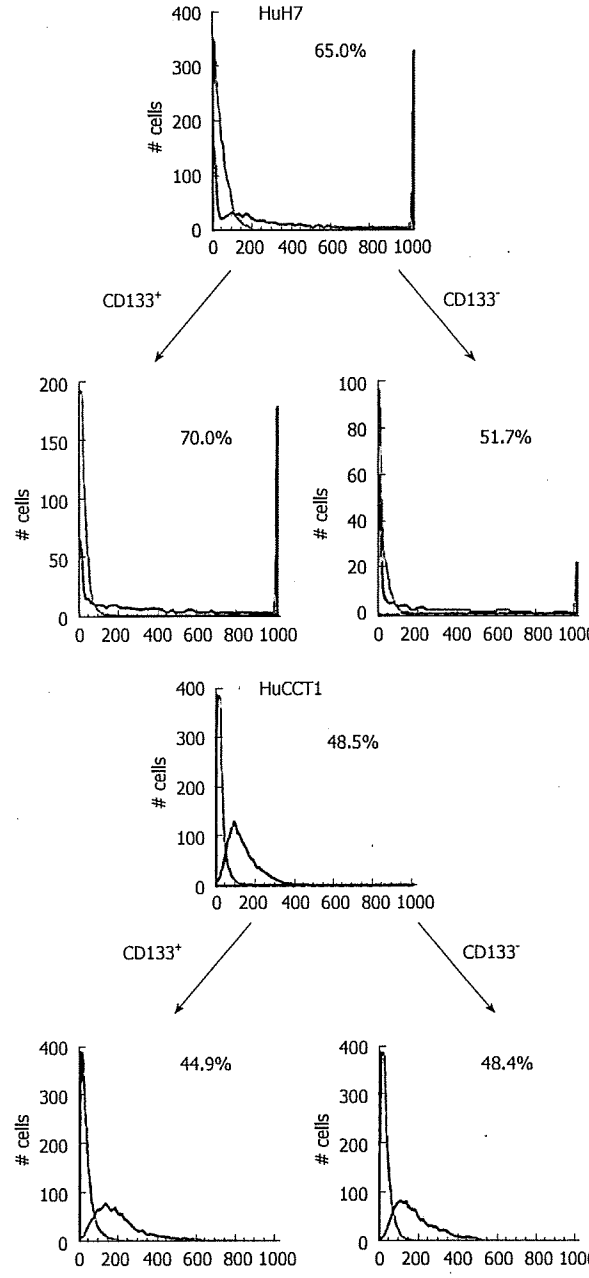

Figure 6 Flow cytometry analysis of CD133 in HuH7 and HuCCT1 cells. CD133$^+$ cells comprised 65.0% of HuH7 and 48.5% of HuCCT1 cells. CD133$^+$ and CD133$^-$ cells could be generated from CD133$^+$ and CD133$^-$ subpopulations of HuH7 and HuCCT1 cells after 4 wk subculture.

CK19$^+$ or CD133$^+$/AFP$^+$ cells were present in both HuH7 and HuCCT1 cells (Figure 5).

Cell sorting of cultured cells with regard to CD133 expression

FACS was performed using two cell lines (HuH7 and HuCCT1). The flow cytometry analysis with regard to CD133 expression is shown in Figure 6. The percentages of CD133$^+$ cells from flow cytometry were 65.0% in HuH7 and 48.5% in HuCCT1 cells. After cell sorting, CD133$^+$ and CD133$^-$ cells derived from HuH7 or HuCCT1 cells were cultured separately for 4 wk. After

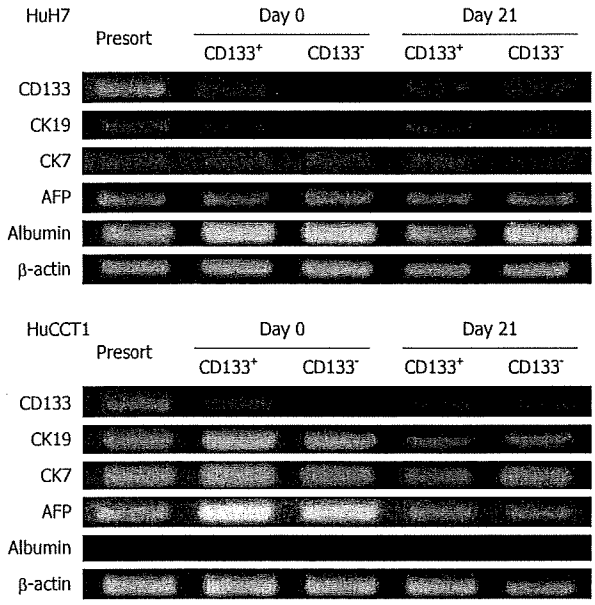

Figure 7 Expression CD133, CK19, CK7, AFP and albumin in HuH7 and HuCCT1 cells before sorting, just after sorting (day 0), and after 3 wk subculture (day 21). At day 0, expression of CD133 mRNA was observed in only the CD133$^+$ population in HuH7 and HuCCT1 cells. However, CD133$^+$ and CD133$^-$ populations expressed similar levels of CD133 mRNA at day 21. At day 21, CD133$^+$ and CD133-HuH7 and HuCCT1 cells showed almost similar mRNA expression patterns.

4 wk subculturing, both CD133$^+$ and CD133$^-$ subpopulations returned to almost the pre-sorting cellular population that comprised both CD133$^+$ and CD133$^-$ cells (Figure 6). These results suggested that CD133$^-$ HuH7 and HuCCT1 cells generated CD133$^+$ and CD133$^-$ progenies during subculture.

Expression patterns of mRNA in CD133$^+$ and CD133$^-$ cells

The expression patterns of CD133, CK19, CK7, AFP and albumin were examined in HuH7 and HuCCT1 cells before sorting, just after sorting (day 0), and after 3 wk subculture (day 21). At day 0, the expression of CD133 mRNA was observed in only the CD133$^+$ population in both HuH7 and HuCCT1 cells. However, CD133$^+$ and CD133$^-$ populations expressed the CD133 mRNA at similar levels at day 21 (Figure 7). These results suggested that CD133$^-$ cells began to express CD133 or produce CD133$^+$ progeny during subculture. Acquisition of CD133 expression in CD133$^-$ cells was consistent with the results of FACS.

On day 0, CK19 was expressed only in CD133$^+$ HuH7 cells. However, CK19 expression was also identified in CD133$^-$ cells at day 21. At day 21, CD133$^+$ and CD133$^-$ HuH7 or HuCCT1 cells showed similar expression patterns for mRNA, except for the slightly more intense expression of CK19 and albumin in CD133$^+$ and CD133$^-$ HuH7 cells, respectively (Figure 7).

Alteration of CD133 expression levels in CD133$^+$ and CD133$^-$ cells

Alterations of CD133 expression levels in HuH7 and

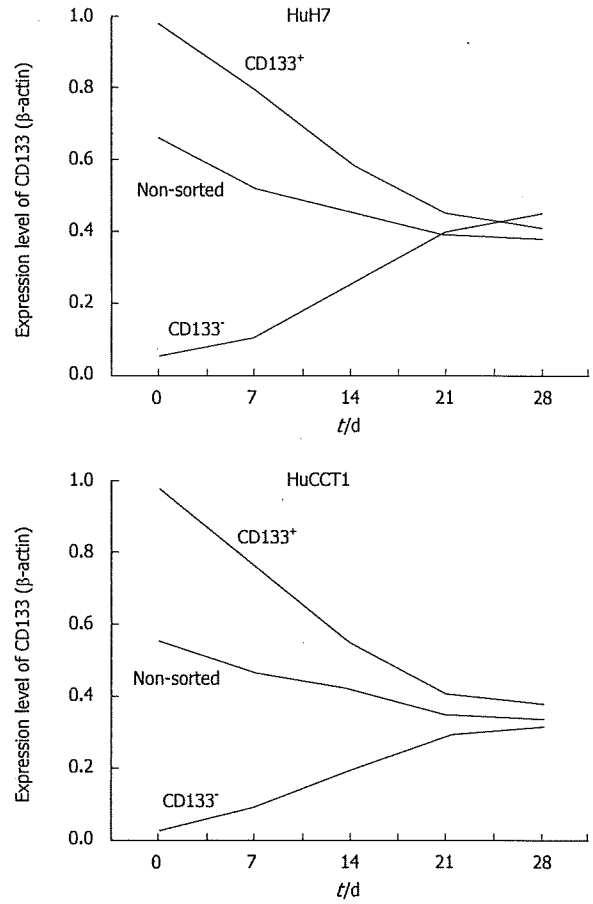

Figure 8 Time-dependent alteration of CD133 expression in HuH7 and HuCCT1 cells. Real-time quantitative RT-PCR revealed CD133 expression levels gradually decreased in non-sorted cells after passage. CD133$^+$ HuH7 and HuCCT1 cells showed decreased expression of CD133. In contrast, CD133 expression increased in CD133$^-$ cells in both cell lines. Around day 21-28, CD133 expression in three types of cells became similar to the level in both cell lines.

HuCCT1 cells were examined by real-time quantitative RT-PCR. HuH7 and HuCCT1 cells showed similar alteration patterns. As shown in Figure 8, CD133 expression levels in non-sorted HuH7 and HuCCT1 cells gradually decreased after passage. CD133 expression levels in CD133$^+$ populations were high just after the sorting (day 0) in both cell lines. These expression levels decreased time-dependently. In contrast, CD133 expression levels in CD133$^-$ cells were very low at day 0, and time-dependently increased. CD133 expression in CD133$^+$ and CD133$^-$ cells reached a similar level around day 21 or 28. In addition, their expression level was also similar to the level of CD133 expression in non-sorted cells at day 21 (Figure 8).

Proliferation assay of CD133$^+$ and CD133$^-$ cells

The proliferation of CD133$^+$ and CD133$^-$ cells were examined using BrdU after 7 d subculture. The percentages of BrdU-labeled cells were as follows: CD133$^+$ HuH7 cells, 22%; CD133$^-$ HuH7 cells, 24%; CD133$^+$ HuCCT1 cells, 39%; and CD133$^-$ HuCCT1 cells, 42%. No significant differences were observed in proliferation of CD133$^+$ and CD133$^-$ HuH7 and HuCCT1 cells.

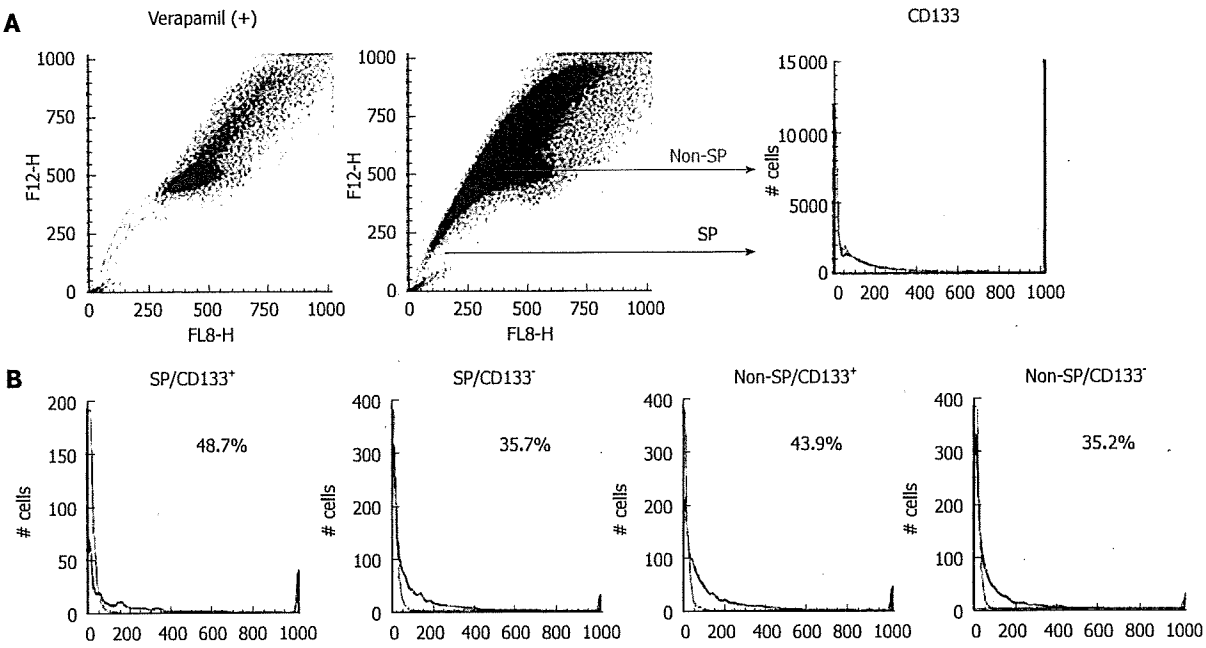

Figure 9 Relationship between CD133$^+$ cells and SP phenotype. A: The percentages of CD133$^+$ cells in SP and non-SP fractions of HuH7 cells were examined using Hoechst 33342 and a PE-conjugated antibody to CD133. The ratio of CD133$^+$ cells was almost the same in the SP and non-SP fractions; B: HuH7 cells were sorted into four populations: SP/CD133$^+$, SP/CD133$^-$, non-SP/CD133$^+$, and non-SP/CD133$^-$. After 4 wk subculture, CD133$^+$ and CD133$^-$ cells were generated at similar levels from all populations.

Relationship between SP and CD133$^+$ cells

Previous studies have reported that CD133$^+$ HCC cells have a greater colony-forming efficiency, higher proliferative activity, and greater ability to form tumors *in vivo*[14-16]. It has been suggested that CD133$^-$ cells are not capable of producing CD133$^+$ cells. However, CD133$^+$ and CD133$^-$ HuH7 cells returned to almost identical cell populations after 4 wk subculture in this study. To resolve this discrepancy, the relationship between SP and CD133$^+$ cells in HuH7 cells was examined because SP is one of the most reliable stem cell markers currently available.

As in the previous study[21], an SP fraction was identified in HuH7 cells. The percentages of CD133$^+$ cells in SP and non-SP fractions were examined using Hoechst 33342 and a PE-conjugated antibody against CD133. The ratios of CD133$^+$ cells were almost the same in both the SP and non-SP fractions (Figure 9). HuH7 cells were sorted into four populations: SP/CD133$^+$, SP/CD133$^-$, non-SP/CD133$^+$, and non-SP/CD133$^-$. Each population was cultured separately for 4 wk and was analyzed again with regard to CD133 expression by FACS. CD133$^+$ and CD133$^-$ cells were produced at similar levels in the four populations (Figure 9). These results suggested no relationship between SP phenotype and CD133 expression.

DISCUSSION

This study involved the histological characterization of CD133$^+$ cells in the liver and the biological characteristics of CD133$^+$ cells derived from human HCC and cholangiocarcinoma cell lines. The results obtained can be summarized as follows. (1) CD133 was expressed constantly in the biliary epithelium in non-neoplastic liver tissues. Most of the CD133$^+$ cells were CK19$^+$ and HepPar-1$^-$ in non-neoplastic liver tissues. (2) In HCC, the expression of CD133 mRNA was observed in all cases by nested RT-PCR, whereas CD133$^+$ cells were identified in only 24% of cases by immunostaining. CD133$^+$ cells were small in number in all the cases of HCC examined. (3) In cholangiocarcinoma, CD133 was expressed diffusely in most carcinoma cells. (4) In combined carcinoma, most of the CD133$^+$ cells were CK19$^+$ and HepPar-1$^-$, although some CD133$^+$ cells were CK19$^-$ and HepPar-1$^+$. (5) In human HCC and cholangiocarcinoma cell lines, CD133$^+$ cells co-expressed CK19 and AFP. (6) CD133$^+$ or CD133$^-$ cells derived from HuH7 and HuCCT1 cell lines similarly produced CD133$^+$ and CD133$^-$ progeny during subculturing. (7) There was no relationship between CD133$^+$ cells and SP phenotype.

In the histological examination, CD133 expression was related closely to CK19 expression. CK19 has been used as not only a biliary marker, but also as a progenitor cell marker. CK19 is expressed usually in the bile ducts, bile ductules, and the canal of Hering[22-24]. Small ductal structures partly surrounded by hepatocytes (the canal of Hering) are currently estimated as hepatic stem/progenitor cells, and these structures are also positive for CD133[25,26]. Before starting this study, it was speculated that CD133 was expressed only in hepatic progenitor cells. However, this study revealed that CD133 is not only a progenitor cell marker, but can also be used as a novel biliary marker.

Some might argue about the discrepancy between the

results with nested RT-PCR and immunohistochemistry for HCC. Nested RT-PCR could detect CD133 expression in all HCC cases; whereas, its expression was observed in only 24% of cases by immunostaining. We speculate that this difference might have been caused by the low expression level of CD133 in HCC. Indeed, non-nested conventional PCR showed CD133 expression in less than half of HCC cases in the preliminary study. That is, it might be difficult to detect CD133 expression in HCC by immunostaining because of the low expression level or the lower number of positive cells.

Other investigators have examined CD133 expression in liver tissues. Yin *et al*[15] have reported that CD133 expression is observed in a small subset of hepatocytes, biliary epithelium, and epithelial clusters in the portal tracts in cirrhotic livers, but CD133 expression is not seen in normal liver[15]. In addition, Ma *et al*[16] have also reported that $CD133^+$ cells are almost absent in non-neoplastic liver tissues. The discrepancy between the previous and current studies might have been cause by the method of immunostaining. Both previous studies used a goat polyclonal antibody and paraffin-embedded specimens, but preliminary trials in the current study could not detect any positive signals for CD133 in paraffin-embedded specimens using any antibodies for CD133; therefore, frozen sections were used instead. In addition, CD133, which is usually expressed on the cellular membrane, was detected in the cytoplasm in previous studies. More recently, Shmelkov *et al*[27] Have examined CD133 in various organs using a unique transgenic mouse model, in which endogenous promoters for CD133 drove the expression of the reporter gene *lacZ*. CD133 was expressed widely in differentiated ductal structures in various organs including bile ducts in the liver[27]. These previous results are consistent with the results of the current study.

CD133 expression was also related closely to CK19 expression in neoplastic liver tissues. In cholangiocarcinoma and combined carcinoma, $CK19^+$ cells constantly co-expressed CD133. $CD133^+$ cells comprised 48.5% of cells in the HuCCT1 cell line, and there were no differences between $CD133^+$ and $CD133^-$ cells in terms of proliferation or mRNA expression. We speculate that CD133 expression in cholangiocarcinoma reflects the biliary phenotype and not the progenitor phenotype. In contrast, some $CD133^+$ cells in HCC and combined carcinoma were $CK19^-$ and $HepPar\text{-}1^+$. $CD133^+/CK19^-/HepPar\text{-}1^+$ cells could not be identified in non-neoplastic livers, although this suggests that $CD133^+$ cells are pluripotent and can differentiate into $CK19^+/HepPar\text{-}1^-$ and $CK19^-/HepPar\text{-}1^+$ cells. In particular, it is interesting that $CD133^+/CK19^-/HepPar\text{-}1^+$ cells are observed in combined carcinoma because the involvement of hepatic progenitor cells is suggested in tumorigenesis of combined carcinoma[28-30].

Until now, some investigators have examined the characterization of $CD133^+$ cells in HCC, and have suggested that $CD133^+$ HCC cells are characterized by higher proliferative activity, expression of "stemness" genes, the ability to self-renew, and greater ability to form tumors *in vivo*[14-16]. They have concluded that $CD133^+$ cells are tumorigenic cancer cells, and located at a higher rank in the cancer-cell hierarchy. However, in the current study, $CD133^+$ HuH7 cells were not different from $CD133^-$ cells in terms of proliferation. In addition, $CD133^-$ cells could generate both $CD133^+$ and $CD133^-$ cells in subcultures, which did not support the existence of a cancer-cell hierarchy with respect to CD133 expression. As noted in previous studies, $CD133^+$ cells comprised about half of the carcinoma cells in the HuH7 cell line (65.0% in the current study, 46.7% or 65.0% in previous studies[14,16]). The percentage of $CD133^+$ cells seems too high to suggest that CD133 cells are tumor-initiating cells. Indeed, $CD133^-$ cells were able to give rise to $CD133^+$ cells in the subculture system in the previous study[16]. Moreover, a more recent study has revealed that CD133 expression is not restricted to stem cells, and $CD133^+$ and $CD133^-$ cells derived from colon cancer are capable of initiating tumors in immunodeficient mice[27].

To resolve the uncertainties regarding CD133 expression and tumor-initiating cells, the relationship between $CD133^+$ cells and SP phenotype was examined in our study. SP is a minor population with extreme tumorigenic potential, and it is supposed that tumor-initiating cells exist in SP cells. If $CD133^+$ cells are tumor-initiating cells, $CD133^+$ cells should be related closely to the SP phenotype, and $CD133^-$ cells should not exist in the SP fraction. However, there was no difference in the $CD133^+/CD133^-$ cellular population between SP and non-SP fractions. In addition, four cell populations ($SP/CD133^+$, $SP/CD133^-$, non-$SP/CD133^+$, and non-$SP/CD133^-$) could similarly produce $CD133^+$ and $CD133^-$ cells during subculture. It is speculated that CD133 expression might reflect the progenitor phenotype in HCC; however, CD133 alone is not sufficient to detect tumor-initiating cells.

It seems important to know that CD133 is one of the progenitor cell markers in the liver, but this is not specific. We have to determine the conditions to identify a pure hepatic progenitor or stem cell population, using multiple surface markers including CD133. From the biliary aspect, CD133 could become a useful marker, because this is a surface antigen. We can use this molecule for sorting or purification of biliary epithelium.

In conclusion, this study revealed that CD133 can be a biliary and progenitor cell marker in liver tissues. However, CD133 alone is not sufficient to detect tumor-initiating cells in cultured cells.

COMMENTS

Background

CD133 is recognized as a stem cell marker for normal and cancerous tissues in various organs. The histological characteristics of hepatic $CD133^+$ cells have not been examined fully, especially in non-neoplastic liver tissues and non-hepatocellular liver cancers.

Research frontiers

Previous studies have shown that CD133 can be used as a maker of cancer stem cells in human hepatocellular carcinoma (HCC). The current study