Cytoplasmic expression of CD133 is an important risk factor for overall survival in hepatocellular carcinoma

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Abstract. CD133 antigen has been used to identify cancer stem cells in several solid tumor types, including hepatocellular carcinomas (HCCs). The aim of this study was to investigate whether the expression and subcellular localization of CD133 correlated with the clinicopathological factors, recurrence, and survival in HCC patients. Tissue specimens from 136 HCC patients who underwent curative primary hepatectomy between 2000 and 2005 were collected and immunohistochemically analyzed for CD133 expression. Positive immunohistochemical results and subcellular localization of CD133 were determined, and the correlation between CD133 expression and clinicopathological factors of HCC patients were evaluated. CD133-positive tumor cells were observed in 30 (22.1%) cases. Cytoplasmic and membranous expressions were observed in 22 (16.2%) and 20 (14.7%) of the CD133-positive cases, respectively. Positive cytoplasmic expression of CD133 was found to be associated with the overall survival of HCC patients, especially in stage III and IVA HCC patients (p=0.0092). Univariate analysis revealed that pre-operative serum albumin, α-fetoprotein (AFP) levels, tumor size, portal venous invasion, and cytoplasmic CD133 expression were important risk factors in HCC. Multivariate analysis revealed that among the factors related to tumor aggressiveness, cytoplasmic expression of CD133 showed the most significant association with overall survival, although the difference was not statistically significant (P=0.0681). Cytoplasmic expression of CD133 was a significant risk factor for the overall survival of HCC patients. Patients with stage III and IVA HCC showing positive cytoplasmic expression of CD133 are more likely to have a worse prognosis.

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Introduction

Hepatocellular carcinoma (HCC) accounts for 5.7% of all cancers, excluding skin tumors, worldwide (1). Because of the very poor prognosis, the number of new HCC cases and the number of deaths from HCC has remained almost the same despite of the recent progress in treatment strategies. Most of the HCC patients who undergo hepatic resection or additional locoregional therapy develop local and remote recurrence (2,3). Ikeda *et al* (4) reported that the hepatocellular carcinogenesis rates for the patients with cirrhosis caused by hepatitis B or hepatitis C virus were 49% at the tenth year, and for those who underwent curative surgical or locoregional therapy, the rate of recurrence was 75% at the fifth year and 89% at the tenth year. The molecular mechanisms underlying the development and progression of HCCs remain unclear.

Recently, the mechanisms of normal stem cells has been applied for cancer cells. Cancer stem cells have the ability to self-renew and differentiate, thereby sustaining tumor growth (5). To date, the existence of cancer stem cells has been confirmed in leukemia (6), as well as in several solid tumors, including brain tumors (7) and breast (8), pancreatic (9), lung (10), and colorectal cancers (11,12). Cancer stem cells might be able to withstand radiation and chemotherapy, due to the preferential expression of resistance molecules or activation of specific signaling pathways (13). CD133 has been used to identify cancer stem cells in various tumors. CD133 antigen is a 5-transmembrane glycoprotein and was originally identified as a cell surface antigen present on $CD34^+$ hematopoietic stem cells (14). It has been shown that CD133-positive cells isolated from HCC cell lines have the ability to efficiently form tumors in mice (15,16), supporting the cancer stem cell hypothesis.

However, the clinicopathological significance of CD133 in HCCs remains controversial (17,18). Therefore, we designed this study to investigate the potential clinical role

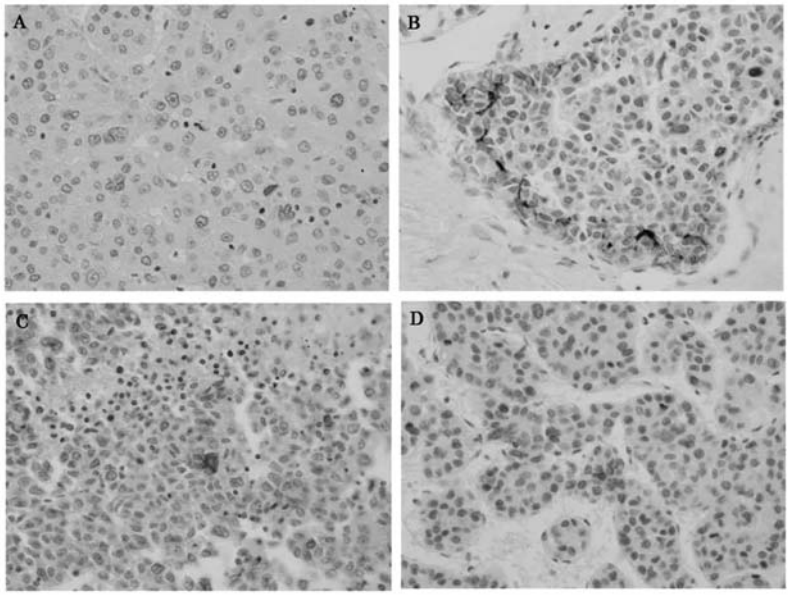

Figure 1. CD133 immunoreactivity in hepatocellular carcinoma. (A) Negative immunostaining for CD133 in tumor cells. (B) Positive membranous immunostaining for CD133 in tumor cells. (C) Diffuse cytoplasmic immunostaining for CD133 in tumor cells. (D) Perinuclear dot-like immunostaining for CD133 in tumor cells. Original magnification, x400.

of the immunohistochemically expressed CD133 in a large series of HCC patients.

Materials and methods

Patients and tissue specimens. Tissue specimens were collected from 136 consecutive HCC patients who underwent primary curative hepatectomy at the First Department of Surgery, Hokkaido University Hospital, during the period between 2000 and 2005. Of the 136 patients, 112 were male, 71 were >60 years old (mean age, 61, range, 35-78), 54 (39.7%) were positive for the hepatitis B virus surface antigen (HBs-Ag) alone, 48 (35.3%) were positive for the hepatitis C virus antibody (HCV-Ab) alone, 28 (20.6%) were negative for both HBs-Ag and HCV-Ab, and 6 (4.4%) were positive for both HBs-Ag and HCV-Ab. As per the macroscopic typing of HCC, which was advocated by the Liver Cancer Study Group in Japan (19), the patients could be classified into the single nodular type, single nodular with extranodular growth type, confluent multinodular type, and infiltrative type. On the basis of these types, we subclassified the patients into 2 groups: nodular and extensive. The single nodular type was considered as the nodular group (80 cases), and the remaining were included in the extensive group (56 cases). The dimensions of each specimen were measured to determine the tumor size. Tumors were staged and graded according to the Liver Cancer Study Group of Japan, 2003 (19). Of the 136 patients, 16 were in stage I, 68 in stage II, 33 in stage III, and 19 in stage IVA. Portal venous invasion, hepatic venous invasion, bile duct invasion, and intrahepatic metastasis were observed in 34 (25.0%), 10 (7.4%), 4 (2.9%), and 40 (29.4%) cases, respectively. In 46 cases, non-cancerous liver tissue showed cirrhosis.

The median follow-up period was 58.5 months (range, 31.6-90.5). Ultrasonography, dynamic computed tomography, magnetic resonance imaging, and laboratory tests

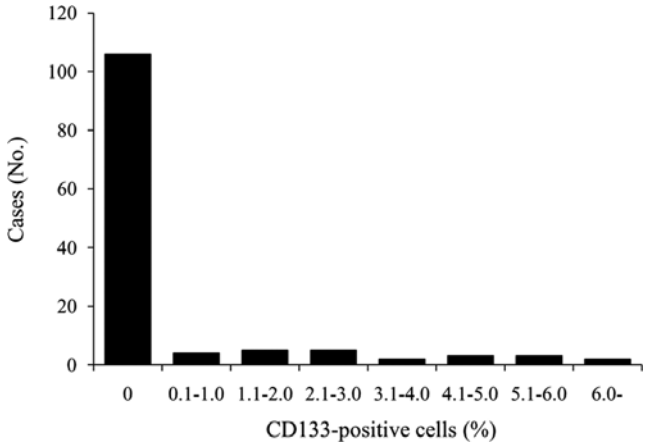

Figure 2. Proportion of CD133-positive tumor cells. CD133-positive tumor cells were counted at high magnification, and then divided by the total number of nuclei of tumor cells counted in the same fields. CD133-positive tumor cells were observed in 30 cases, and the proportion of these cells was 0-9.4%, with the median proportion of 0.64%.

for (α-fetoprotein (AFP), the lens culinaris agglutinin-reactive fraction of AFP (AFP-L3), and protein induced by vitamin K absence or antagonists (PIVKA-II) were performed every 3 months. HCC recurrence was observed in 93 patients. Informed consent was obtained from each patient according to the Ethics Committee Guidelines of our institution.

Immunohistochemistry. Tissues were fixed in 10% formalin, embedded in paraffin, cut into 3-4-μm sections, mounted on silane-coated slides, and dried at 58˚C for 30 min. One section from each tissue was stained with hematoxylin and eosin for histological examination. Immunohistochemical staining was performed on automated immunostainer

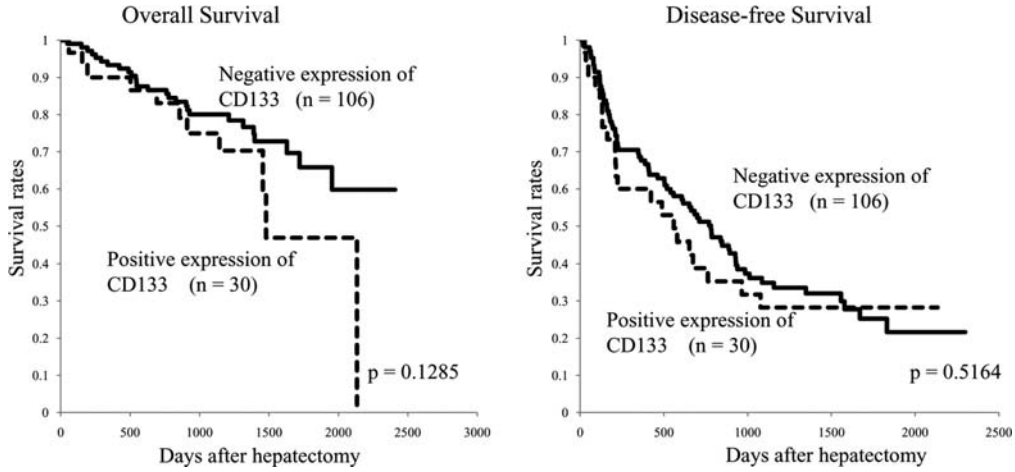

Figure 3. Overall survival and disease-free survival curves of hepatocellular carcinoma patients according to the status of CD133 expression. The differences in survival rates of cases with positive CD133 expression and those with negative CD133 expression were evident, but were not statistically significant. There was no association of disease-free survival between patients showing positive and those showing negative expression of CD133.

(NexES®; Ventana Medical Systems, Tucson, AZ, USA) using the Ventana iVIEW DAB Universal kit (Ventana). The slides were deparaffinized with xylene and rehydrated through graded concentrations of ethanol. After the slides were deparaffinized, antigen retrieval was performed for 2.5 min in a pressure cooker using 1 mmol/l ethylenediamine tetraacetic acid solution (pH 8.0). Endogenous peroxidase was blocked with 3% H_2O_2 for 4 min at 37˚C. Subsequently, the slides were rinsed and incubated at 37˚C for 32 min with a 1:300 dilution of a rabbit polyclonal anti-CD133 primary antibody (Abcam, Inc., Cambridge, UK). Signal enhancement was performed using the Ventana Amplification kit (Ventana) for 2 incubations of 8 min each. Slides were then incubated with iVIEW (Ventana) biotinylated goat anti-rabbit immunoglobulin (Ig) G and IgM secondary antibodies for 8 min, followed by incubation in a blocker for 4 min. Subsequently, the slides were counterstained with hematoxylin for 1 min and rinsed. After the slides were removed from the instrument, they were manually dehydrated and coverslipped.

CD133-positive tumor cells were counted in 10 random and non-overlapping fields at high magnification (x400). The results were expressed as the percentage of the total number of tumor cell nuclei in the fields that showed maximum CD133-positive tumor cells. For statistical analysis, patients were divided into negative (percentage of CD133-positive tumor cells, 0) versus positive (percentage of CD133-positive tumor cells >0) CD133 expression. The entire sections were also screened for features such as cytoplasmic/membranous CD133 expression in tumor cells and that in non-cancerous hepatocytes or the bile duct epithelial cells in the background.

Statistical analysis. Statistical analyses were performed using the StatView J 5.0 software package for Windows (SAS Institute Inc., Cary, NY). Cumulative survival and disease-free survival rates were calculated using the Kaplan-Meier method, and comparisons between groups were performed by using the log-rank test. The Cox proportional hazards model was used for multivariate analysis. Statistical calculations using standard tests (χ^2 and t-tests) were performed where appropriate. Significance was defined as a P-value of <0.05.

Results

CD133 expression in HCCs. Representative staining images of HCCs are shown in Fig. 1. In the overall series, CD133-positive tumor cells were observed in 30 (22.1%) cases, with a median proportion of 0.64 (range: 0-9.4) (Fig. 2). Furthermore, cytoplasmic and membranous expressions of CD133 were identified in 22 (16.2%) and 20 (14.7%) CD133-positive cases, respectively. There were two patterns of cytoplasmic positivity, diffuse (Fig. 1C) or perinuclear dot-like (Fig. 1D). None of the non-cancerous hepatocytes showed positive immunoreactivity for CD133. CD133 positivity was seen at the endoluminal surface of the bile duct epithelial cells in the background. Positive expression of CD133 was significantly associated with elevated serum AFP levels and histologically high-grade tumor (Table I). Positive cytoplasmic expression of CD133 also significantly correlated with elevated serum AFP levels and histologically high-grade tumor (Table II). With regard to the primary recurrent site, remote metastasis was significantly more frequent in cases showing positive membranous expression of CD133. Portal venous invasion, which included microscopic and macroscopic invasion, was not significantly correlated with CD133 expression. However, both cytoplasmic and membranous expression of CD133 were associated with tumors involving a major branch of the portal vein (P=0.0221, 0.0104, respectively).

Overall survival and CD133 expression. Of the 136 patients, 39 died; 33 due to HCC, 3 each due to liver failure and another disease. There was no difference in the overall survival between the cases that showed positive and those that showed negative CD 133 expression (Fig. 3), while the difference in survival rates was significant between the patients with positive and negative cytoplasmic CD133 expression (Fig. 4). The 1- and 3-year survival rates of patients with positive cytoplasmic expression of CD133 were 86.4

Table I. Comparative analysis of the clinicopathological findings between CD133-positive and CD133-negative groups in HCC patients.

Clinical factors	CD133+ (n=30)	CD133- (n=106)	p-value
Age			
≤60/>60	15/15	50/56	0.7841
Gender			
Male/Female	23/7	89/17	0.3548
HBsAg			
+/-	17/13	43/63	0.1169
HCVAb			
+/-	11/19	43/63	0.7000
Albumin			
≤4.0/>4.0 (g/dl)	10/20	46/60	0.3228
Total bilirubin			
≤0.8/>0.8 (mg/dl)	25/5	72/34	0.0994
ICGR15			
≤15/>15 (%)	18/12	57/49	0.5449
AFP			
≤200/>200 (ng/ml)	16/14	78/28	0.0340
PIVKA-II			
≤100/>100 (mAU/ml)	13/17	57/48	0.2897
Recurrence			
Yes/No	21/9	72/34	0.8291
Primary recurrence site			
Liver/Remote	14/7	58/14	0.1804
Anatomic resection[a]			
Yes/No	23/7	74/32	0.4636
Tumor morphology[b]			
Nodular/Extensive	15/15	65/41	0.2660
Tumor number			
Single/Multiple	18/12	76/30	0.2208
Tumor size			
≤10/>10 (cm)	23/7	94/12	0.0938
Tumor grade			
Well/Mod/Por	1/10/19	6/78/21[c]	<0.0001
Portal venous invasion			
+/-	11/19	23/83	0.0946
Hepatic venous invasion			
+/-	4/26	6/100	0.1552
Bile duct invasion			
+/-	2/28	2/104	0.1713
Intrahepatic metastasis			
+/-	11/19	29/77	0.3232
Cirrhosis			
+/-	11/19	35/71	0.7093

[a]Segmentectomy, lobectomy, or extended lobectomy. [b]Nodular type included single nodular type. [c]Tumor histology was unknown in one case. HBsAg, hepatitis B virus antigen; HCVAb, hepatitis C virus antibody; ICG R15, indocyanine green retention rate at 15 min; AFP, α-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists; mod, moderate; por, poor.

and 64.4%, respectively, while those of patients with negative cytoplasmic expression of CD133 were 93.8 and 66.6%, respectively. Membranous expression of CD133 had no correlation with the overall survival: the 1- and 3-year survival rates of the patients with positive membranous expression of CD133 were 95.0 and 78.8%, respectively, while those of patients with negative membranous expression of CD133 were 95.0 and 79.7%, respectively (P=0.5054). Univariate analysis revealed that preoperative serum albumin, AFP level, PIVKA-II level, tumor number, tumor size, portal venous invasion, hepatic venous invasion, intrahepatic metastases, and cytoplasmic expression of CD133 were significant risk factors (Table III). Multivariate analysis revealed that preoperative albumin level, AFP level, PIVKA-II level, and portal venous invasion, but not cytoplasmic expression of CD133, were independent risk factors for patient survival (Table IV).

The differences in the survival rate of the 52 cases with stage III and IVA HCC were more significant between patients showing positive and those showing negative cytoplasmic expression of CD133 (P=0.0092), compared to the differences in patients with stage I, II, III, and IVA HCC (Fig. 5). In the patients with stage III and IVA HCC, the 1- and 3-year survival rates of cases showing positive cytoplasmic expression of CD133 were 70.0 and 16.7%, respectively, while those showing negative cytoplasmic expression were 82.9 and 69.4%, respectively. Univariate analysis revealed that preoperative serum albumin, AFP level, tumor size, portal venous invasion, and cytoplasmic expression of CD133 were important risk factors (Table V). Multivariate analysis revealed that preoperative albumin level was the only independent risk factor for patient survival (Table VI). Among the factors related to tumor aggressiveness, cytoplasmic expression of CD133 was found to be most significantly associated with overall survival, although the difference was not statistically significant (P=0.0681).

Disease-free survival and CD133 expression. The difference in the disease-free survival was not significant between

Table II. Comparative analysis of the clinicopathological findings and CD133 status in HCC patients.

	CD133 cytoplasmic			CD133 menbranous		
Clinical Factors	Positive (n=22)	Negative (n=114)	p-value	Positive (n=20)	Negative (n=116)	p-value
Age ≤60/>60	10/12	55/59	0.8104	14/6	51/65	0.0313
Gender Male/Female	17/5	95/19	0.4948	16/4	96/20	0.7650
HBsAg +/-	13/9	47/67	0.1224	13/7	47/69	0.0417
HCVAb +/-	9/13	45/69	0.8997	6/14	48/68	0.3368
Albumin ≤4.0/>4.0 (g/dl)	9/13	47/67	0.9778	7/13	49/67	0.5434
Total bilirubin ≤0.8/>0.8 (mg/dl)	17/5	80/34	0.5004	17/3	80/36	0.1431
ICGR15 ≤15/>15 (%)	14/8	61/53	0.3819	12/8	63/53	0.6366
AFP ≤200/>200 (ng/ml)	10/12	84/30	0.0087	12/8	82/34	0.3393
PIVKA-II ≤100/>100 (mAU/ml)	9/13	61/52	0.2615	9/11	61/54	0.5064
Recurrence Yes/No	17/5	76/38	0.3273	13/7	80/36	0.7247
Primary recurrence site Liver/Remote	10/7	59/17	0.9176	7/6	65/15	0.0284
Anatomic resection[a] Yes/No	16/6	81/33	0.8737	16/4	81/35	0.3529
Tumor morphology[b] Nodular/Extensive	11/11	69/45	0.3584	11/9	69/47	0.7068
Tumor number Single/Multiple	12/10	82/32	0.1061	13/7	81/35	0.6661
Tumor size ≤10/>10 (cm)	16/6	101/13	0.0493	16/4	101/15	0.3997
Tumor grade Well/Mod/Por	1/7/14	6/81/26[c]	0.0006	0/7/13	7/81/27[c]	0.0007
Portal venous invasion +/-	8/14	26/88	0.1788	8/12	26/90	0.0935
Hepatic venous invasion +/-	3/19	7/107	0.2175	3/17	7/109	0.1560
Bile duct invasion +/-	1/21	3/111	0.6267	1/19	3/113	0.5552
Intrahepatic metastasis +/-	10/12	30/84	0.0713	6/14	34/82	0.9502
Cirrhosis +/-	10/12	36/78	0.2079	7/13	39/77	0.9042

[a]Segmentectomy, lobectomy or extended lobectomy. [b]Nodular type included single nodular type. [c]Tumor histology was unknown in one case. HBsAg, hepatitis B virus antigen; HCVAb, hepatitis C virus antibody; ICG R15, indocyanine green retention rate at 15 min; AFP, α-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists; mod, moderate; por, poor.

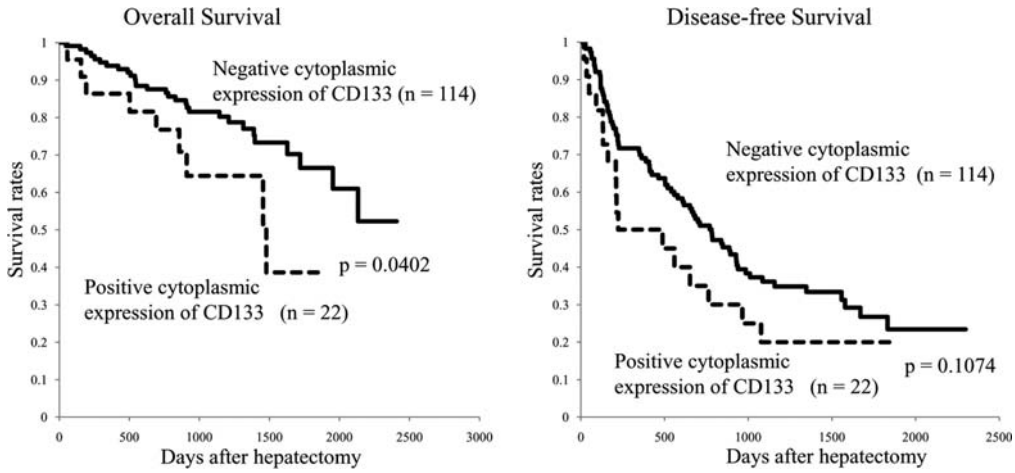

Figure 4. Overall survival and disease-free survival curves of hepatocellular carcinoma patients with positive and negative cytoplasmic expression of CD133. Overall survival rates of patients with negative cytoplasmic expression of CD133 were significantly higher than those with positive expression of CD133. The differences in disease-free survival rates between cases with positive versus those with negative cytoplasmic expression of CD133 were evident, but not statistically significant.

Table III. Univariate analysis of the factors affecting overall survival and disease-free survival.

	No.	Overall survival p-value	Disease-free survival p-value
Age (year)			
≤60/>60	65/71	0.6365	0.0083
Gender			
Male/Female	112/24	0.2555	0.9671
HBsAg			
+/-	60/76	0.0992	0.0003
HCV			
+/-	54/82	0.5323	0.0715
Albumin (g/dl)			
≤4.0/>4.0	56/80	0.0009	0.0020
Total bilirubin (mg/dl)			
≤0.8/>0.8	97/39	0.1546	0.4222
ICGR15 (%)			
≤15/>15	75/61	0.7760	0.1301
AFP (ng/ml)			
≤200/>200	94/42	<0.0001	0.1247
PIVKA2 (mAU/ml)			
≤100/>100	70/65	0.0005	0.0064
Tumor morphology			
Nodular/Extensive	80/56	0.9848	0.5315
Tumor number			
Single/Multiple	94/42	0.0014	<0.0001
Tumor size (cm)			
≤10/>10	117/19	0.0005	<0.0001
Histology			
Well + Mod/Por	95/40	0.1011	0.0091
Portal vein invasion			
+/-	34/102	<0.0001	<0.0001
Hepatic vein invasion			
+/-	10/126	0.0010	<0.0001
Intrahepatic metastasis			
+/-	40/96	0.0110	<0.0001
Cirrhosis			
+/-	46/90	0.2779	0.0656
CD133 cytoplasmic			
+/-	22/114	0.0402	0.1074

HBsAg, hepatitis B virus antigen; HCVAb, hepatitis C virus antibody; ICG R15, indocyanine green retention rate at 15 min; AFP, α-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists; mod, moderate; por, poor.

patients with positive and those with negative CD133 expression (Fig. 3). Positive membranous expression of CD133 and positive cytoplasmic expression of CD133 were not associated with disease-free survival (P=0.7429, P=0.1074, respectively). Although the difference was not

Table IV. Multivariate analysis of the factors affecting overall survival.

	p-value	Risk ratio	95% Confidence interval
Albumin ≤4.0 (g/dl)	0.0016	3.663	1.634-8.929
AFP >200 (ng/ml)	0.0017	3.322	1.571-7.024
PIVKA2 >100 (mAU/ml)	0.0175	2.793	1.197-6.515
Tumor number multiple	0.9589	1.025	0.398-2.639
Tumor size >10 (cm)	0.4047	0.641	0.225-1.826
Portal vein invasion (+)	0.0313	2.433	1.083-5.464
Hepatic vein invasion (+)	0.7043	1.312	0.323-5.348
Intrahepatic metastasis (+)	0.6651	1.238	0.470-3.257
CD133 cytoplasmic (+)	0.3108	1.550	0.664-3.610

AFP, α-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists.

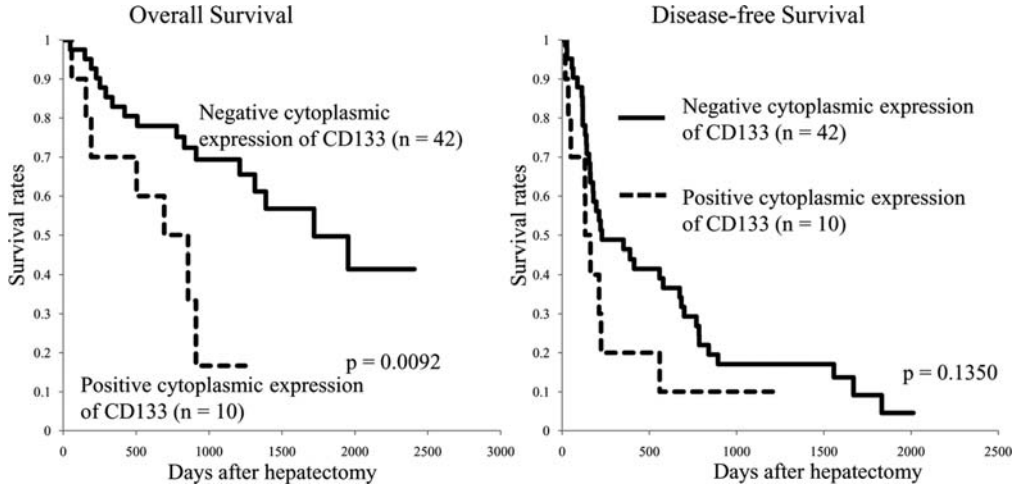

Figure 5. Overall survival and disease-free survival curves in hepatocellular carcinoma patients in stage III and IVA according to the cytoplasmic expression patterns of CD133. The differences were more significant in survival rates between patients with positive and those with negative cytoplasmic expression of CD133, compared to the difference in patients with stage I, II, III, and IVA HCC. The differences in the disease-free survival rates between patients with CD133-positive and those with CD133-negative cells were evident, but not statistically significant.

significant, patients with negative cytoplasmic expression showed a higher disease-free survival rate: the 1- and 3-year disease-free survival rates of the patients with positive cytoplasmic expression of CD133 were 50.0% and 20.0%, respectively, while those of patients with negative cytoplasmic expression of CD133 were 69.0 and 36.1%, respectively (Fig. 4).

Univariate analysis revealed that age, HBV infection, preoperative serum albumin, PIVKA-II level, tumor number, tumor size, histological grade of tumor, portal venous invasion, hepatic venous invasion, and intrahepatic metastases were significant risk factors (Table III). Multivariate analysis revealed that preoperative albumin level (P=0.0214, risk ratio, 1.792, 95% confidence interval, 1.091-2.950) and tumor size (P=0.0328, risk ratio, 2.237, 95% confidence interval, 1.068-4.685) were independent risk factors for disease-free survival.

In the 52 cases with stage III and IVA HCCs, preoperative serum albumin, PIVKA-II level, tumor size, histological tumor grade, portal venous invasion, and hepatic venous invasion were found to be significant risk factors by univariate analysis (Table V), and preoperative albumin level (P=0.0180, risk ratio, 2.381, 95% confidence interval, 1.160-4.878) and PIVKA-II level (P=0.0174, risk ratio, 2.506, 95% confidence interval, 1.175-5.346) were found to be independent risk factor for disease-free survival by multivariate analysis.

Discussion

The present study shows that cytoplasmic expression of CD133 in HCC is associated with elevated serum AFP level, histologically high-grade tumor, and tumor invasion to the major branch of the portal vein. Moreover, positive

Table V. Univariate analysis of the factors affecting overall survival and disease-free survival in stage III and IVA HCC patients.

	No.	Overall survival p-value	Disease-free survival p-value
Age (year)			
≤60/>60	26/26	0.8781	0.1747
Gender			
Male/Female	43/9	0.1822	0.6316
HBsAg			
+/-	29/23	0.5165	0.0604
HCV			
+/-	16/36	0.6117	0.8400
Albumin (g/dl)			
≤4.0/>4.0	24/28	0.0268	0.0082
Total bilirubin (mg/dl)			
≤0.8/>0.8	40/12	0.5036	0.9727
ICGR15 (%)			
≤15/>15	34/18	0.5185	0.7273
AFP (ng/ml)			
≤200/>200	26/26	0.0145	0.3582
PIVKA2 (mAU/ml)			
≤100/>100	18/34	0.0903	0.0099
Tumor morphology			
Nodular/Extensive	24/28	0.9489	0.1575
Tumor number			
Single/Multiple	15/37	0.6983	0.4865
Tumor size (cm)			
≤10/>10	38/14	0.0137	0.0002
Histology			
Well + Mod/Por	33/19	0.1539	0.0462
Portal vein invasion			
+/-	27/25	0.0196	0.0141
Hepatic vein invasion			
+/-	10/42	0.1389	0.0442
Intrahepatic metastasis			
+/-	35/17	0.4994	0.2392
Cirrhosis			
+/-	19/33	0.6495	0.8355
CD133 cytoplasmic			
+/-	10/42	0.0092	0.1350

HBsAg, hepatitis B virus antigen; HCVAb, hepatitis C virus antibody; ICG R15, indocyanine green retention rate at 15 min; AFP, α-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists; mod, moderate; por, poor.

cytoplasmic expression of CD133 was found to be an important risk factor for overall survival, especially in patients with stage III and IVA HCC.

The study demonstrated that CD133 expression is associated with the clinicopathological factors, including preoperative serum AFP level and poorly differentiated tumors. The results are consistent with those of another study (17), with respect to the association between patients with CD133 positive and negative expression for overall survival and the difference in elevated serum AFP level and high-grade tumor. Our results were different with regard to the association between CD133 expression and disease-free survival. However, Salnikov *et al* (18) reported that there was no correlation between the amount of CD133 positive cells and clinicopathological status of HCC patients. Moreover, in these previous studies, almost all cases had CD133-positive tumor cells, whereas in our study, not all the cases were CD133 positive. The discrepancies may be have arisen due to the differences in the antibody used, the quality of tissue samples analyzed, the immunostaining protocols and scoring systems, the number of cases, and the difference in the tumor grading and staging of the participants. Although several studies showed that CD133-positive cells separated from HCC cell lines have the ability to self-renew and proliferate, suggesting that these cells are putative stem/progenitor cells (15,16,20), further investigation is required to reveal the significance and clinical impact of CD133 expression in HCC patients.

CD133 antigen was identified as a cell surface antigen on $CD34^+$ hematopoietic stem cells (14). CD133 localizes to the plasma membrane protrusions at the apical surface of cells (21). However, in normal retina and some epithelial and non-epithelial malignancies, cytoplasmic localization of CD133 was observed (22,23). Diffuse cytoplasmic staining was observed in the retina and gastrointestinal stromal tumors, and perinuclear dot-like staining pattern was observed in the glioblastoma multiforme, myelogenous leukemia, pancreatic ductal adenocarcinoma, and ovarian cancer. Cytoplasmic expression of CD133 was associated with the patient survival in ovarian cancers, although the difference was not statistically significant (23). There was no description about the subcellular localization of CD133 in HCC, and this is the first study analyzing the expression and subcellular distribution of CD133 antigen in HCC tissues from a large series of patients who underwent curative hepatic resection. We found that CD133 expression might be observed both in the cytoplasm and membrane of tumor cells, and the correlation of CD133 expression to patient survival was clearly demonstrated.

Any protein having different subcellular localization may have a specific function, although the physiological role of CD133 is yet unknown. CD133 has splice variants in glial cells (24), and abnormal DNA methylation of CD133 has been observed in colorectal tumors and glioblastomas (25). Molecular genetic analysis of autosomal recessive retinal degeneration indicated that the affected individuals had a frameshift mutation in prominin 1 (synonym for CD133) with premature termination of translation, and the truncated protein cannot be transported to the cell surface (26). Absence of membranous CD133 expression, as observed in HCCs

Table VI. Multivariate analysis of the factors affecting overall survival in stage III and IVA HCC patients.

	p-value	Risk ratio	95% Confidence interval
Albumin ≤4.0 (g/dl)	0.0338	2.577	1.075-6.173
AFP >200 (ng/ml)	0.1348	2.060	0.799-5.315
Tumor size >10 (cm)	0.7332	1.189	0.439-3.221
Portal vein invasion (+)	0.2675	1.745	0.652-4.673
CD133 cytoplasmic (+)	0.0681	2.604	0.931-7.299

AFP, α-fetoprotein.

in this study, may probably indicate the accumulation of truncated CD133 protein, internalization of CD133 protein on membranes that may result in rapid degradation. This phenomenon has been already observed for the epidermal growth factor receptor in cultured cells (27). The failure of transportation and insertion of newly synthesized molecules within the Golgi apparatus into the membranes is also discussed as a possible mechanism (28).

In this study, a significant association was observed between cytoplasmic expression of CD133 and overall survival of patients with HCC in advanced stage. There are two principal characteristics that contribute to the high HCC recurrence rate: multicentric carcinogenicity and hematogenous metastasis to the liver and remote organs (4,29). The prognosis of small HCCs after hepatic resection differs depending on the presence of liver cirrhosis. Most intrahepatic recurrences of small HCCs after hepatectomy are considered to be *de novo* metachronous tumors (30). Multicentric carcinogenesis due to liver cirrhosis in the remnant liver is an important factor for the postresection survival of patients in an earlier stage of HCC (31). The disease-free survival of patients who underwent resection of large HCCs was shorter than that of the patients with small HCCs (32). Because vascular invasion was more frequently observed in patients with large HCCs, the contribution of hematogenous spread of tumor cells was comparatively higher in the patients with HCC in advanced stage. It has been reported that the recurrence of HCCs might be caused by hematogenous spread of tumor cells among the patients exceeded the Milan criteria, these patients may have unfavorable prognosis compared to those within the Milan criteria who developed the recurrence of HCC that might be caused by *de novo* metachronous tumors (33). As per the cancer stem cell theory, hematogenous metastasis in HCC cases might be more related with recurrence induced by the cancer stem cell, because cytoplasmic expression of CD133 was associated with tumor aggressiveness; elevated serum AFP level, histologically high-grade tumor, and vascular invasion, whereas multistep dedefferentiation would be considered important for multicentric carcinogenesis. Cytoplasmic, not membranous, staining of CD133 may be a useful marker of cancer stem cell in HCC. Further investigation is warranted in order to conclude that subcellular localization of CD133 is a key feature of recognition of cancer stem cells in certain cancers.

In conclusion, positive cytoplasmic expression of CD133 represented the risk of poor prognosis, especially in patients with advanced stage HCC. Cytoplasmic expression of CD133 can serve as a marker for clinical prognosis of HCC patients.

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