CD44 and CD133 Expressions in Primary Tumor Cells Correlate to Survival of Pancreatic Cancer Patients

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Abstract: *Background*: Recent studies have suggested that CD44 and/or CD133 expressing pancreatic cancer cells have potential abilities of self-renewal, tumorigenesis and tumor metastasis. The aim of this study was to investigate whether CD44 and CD133 expressions in primary tumor cells correlate to the survival and clinicopathological findings of pancreatic cancer patients.

Methods: Pancreatic head carcinoma specimens from 80 patients who underwent surgical resection were immunohistochemically assessed for CD44 and CD133 expressions.

Results: Of the 80 specimens, 34 (42.5 %) and 48 (60%) specimens were immunohistochemically positive for CD44 and CD133 expression, respectively. CD44 was expressed on the cell surface and CD133 expression was observed in the cytoplasm of the positive cells, which were located at the peripheral adenocarcinoma glandular structures. There was no significant difference in the 5-year survival rate of patients based on CD44 expression, but the 5-year survival rate of CD133-positive patients was significantly lower than that of CD133-negative patients ($P = 0.0002$). Multivariate analysis revealed that CD44-positive and CD133-negative expression was a favorable prognostic indicator ($P = 0.0424$).

Conclusions: CD44 and CD133 expressions are association with survival and malignant behavior in pancreatic cancer patients.

Keywords: Pancreatic cancer, cancer stem cell, adhesion molecule, CD44, CD133, prognosis.

INTRODUCTION

Patients with pancreatic cancer have a shorter expected survival than patients with any other gastrointestinal malignancy. One principal reason for the poor prognosis of pancreatic adenocarcinoma patients is a strong propensity for the lymphatic and blood-borne dissemination of tumor cells [1]. Recent studies of human pancreatic cancers have shown a population of pancreatic cancer stem cells that are resistant to standard chemotherapy and radiation, and have potential abilities of self-renewal, tumorigenesis and tumor metastasis [2].

CD44 is a transmembrane glycoprotein with roles in lymphocyte homing, hyaluronan degradation, cell-cell interactions, and adhesion [3]. CD44 is expressed in multiple forms, with the most common form, termed "CD44 standard (CD44)". In addition, generation of multiple splice isoforms, termed "CD44 variants (CD44v)", is often seen [4]. In one report, all CD44v were present in pancreatic cancer cases [5, 6]. Interestingly, of CD44v, v2/v6 expression was associated with not only a poorer prognosis [7] but also favorable prognosis [8]. On the other hand, several reports indicated that loss of CD44 expression can be considered a predictor of a poor prognosis in some types of tumors, including tongue cancer [9], laryngeal cancer [10], and colorectal cancer [11]. However, another study reported conflicting findings, demonstrated that positive CD44 expression can be the most important indicator of poor prognosis in patients with colorectal cancer [12].

CD133 is a highly conserved antigen and the human homolog of mouse Prominin-1, and was first identified as a 5-transmembrane cell-surface glycoprotein expressed in a subpopulation of CD34+ hematopoietic stem and progenitor cells derived from human fetal liver and bone marrow [13, 14]. CD133 expression has been detected in several normal tissues, including neuroepithelium and both embryonic and adult immature epithelia [15, 16]. Since a tight correlation between CD133 and CSCs was first documented in hematological tumors [17], CD133 expression has been detected in various types of solid tumors, including brain tumors [18, 19], prostate cancer [20], kidney cancer [21], melanoma [22], ovarian cancer [23], hepatocellular carcinoma [24, 25], colon cancer [26, 27], and pancreatic cancer [28].

In the present study, in an attempt to identify CSCs in solid tumors, we employed immunohistochemical analysis to

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correlate the relationship between CD44 and CD133 expression in terms of clinicopathological findings and survival of pancreatic cancer patients.

MATERIALS AND METHODS

Patients and Specimens

Formalin-fixed, paraffin-embedded blocks of tumor tissue were obtained from 80 patients (52 male and 28 female) who underwent surgical resection at Kagoshima University Hospital for invasive ductal adenocarcinoma of the pancreatic head. All patients underwent macroscopically curative resection by total pancreatectomy, pancreaticoduodenectomy, or pylorus-preserving pancreaticoduodenectomy with lymph node dissection. Patients did not receive any preoperative chemotherapy or radiotherapy. Cancer tissue specimens were collected from the patients after informed consent was obtained, in accordance with the institutional guidelines of our hospital. Patient age ranged from 42-80 years with a mean of 66.0 years. The number of patients with pT1, pT2, pT3, and pT4 tumors was 3 (3.8%), 4 (5.0%), 65 (81.3%), and 8 (10.0%), respectively.

All of the resected primary tumors and lymph nodes were subjected to standard hematoxylin and eosin staining and classified according to the tumor-node-metastasis classification system [29]. Histologically, all of the tumors were invasive ductal adenocarcinomas (35 well differentiated, 42 moderately differentiated, and 3 poorly differentiated). Of 80 tumors examined, lymphatic and venous invasion was observed in 69 (86.3%) and 61 (76.3%), respectively. Lymph node metastasis was present in 51 cases (63.8%).

All patients were assessed by radiography, ultrasonography, and computed tomography every 3 months after discharge. New lesions detected by imaging were considered indicative of relapse. The median follow-up period was 20 months (range: 6-168 months). During this period, 29 patients (36.3%) experienced a recurrence in the liver.

Immunohistochemistry

Primary lesions were fixed in 10% formaldehyde and routinely embedded in paraffin. Five 3-μm-thick sections were cut from the paraffin blocks every 30 μm. Sections were deparaffinized in xylene, rehydrated in a graded series of ethanol, and incubated in 3.0% hydrogen peroxide in methanol for 10 minutes to block endogenous peroxidase. The slides were heated in 10 mM sodium citrate (pH 6.0) for 10 minutes at 100°C in a microwave oven and cooled to room temperature. After incubation in 1% bovine serum albumin for 30 minutes at room temperature, the sections were incubated overnight at 4°C with goat anti-CD133 polyclonal antibody (clone K-18, Santa Cruz Laboratory, Santa Cruz, CA, USA; diluted 1: 200 in phosphate-buffered saline [PBS]) and mouse anti-CD44 monoclonal antibody (clone G44-26, BD Pharmingen, San Jose, CA, USA; diluted 1: 50 in PBS). The reactions were developed using the avidin-biotin immunoperoxidase technique (ABC method). Immunoreactivity was visualized using the Vectastain Elite ABC kit and a 3, 3'-diaminobenzidine solution (Vector Laboratories, Inc., Burlingame, CA, USA). Sections were then briefly counterstained with hematoxylin. For the negative control, sections were incubated with normal goat serum instead of the primary antibody and were then treated with the secondary antibody. All immunostained slides were inspected by two independent observers (S. M. and S. T.), who had no prior knowledge of the clinicopathological findings. Ten fields were evaluated within the body of the tumor and in the area exhibiting tumor invasion, and expression was evaluated in 1000 tumor cells (100 cells per field) with high-power (× 200) microscopy. Samples were considered positive if more than 10% of the tumor cells stained positively for CD44 antibody. Specimens were defined as positive for CD133 expression when positive staining was noted not only on the endoluminal cell membrane but also in the cytoplasm of the tumor cells.

Statistical Analysis

Group differences were statistically analyzed using the χ^2 test. The Kaplan-Meier method was used to analyze survival, and the log rank test was used to estimate differences in survival. Prognostic factors were examined using univariate analysis, multivariate analysis, the Cox proportional hazards regression model. *P*-values less than 0.05 were considered statistically significant. All statistical analysis was performed using the StatView statistical software version 5.0 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Expression of CD44 and CD133 in Pancreatic Head Carcinoma Specimens

The expression of CD44 on the cell surface of cancer cells was seen in 34 (42.5%) of 80 patients (Fig. **1**). Forty-eight (60.0%) patients had CD133-positive cells present in the peripheral site (facing the interstitial space) of the tumor glandular structures. Notably, expression of CD133 was present in the cytoplasm of carcinoma cells (Fig. **1**). Pancreatic cancer cells were divided into 4 categories; 1) the CD44 (+) cells were often negative for staining with anti-CD133 antibody (compare Fig. **1A** and **a**); 2) some CD133 (+) cells were negative for CD44 expression (compare Fig. **1B** and **b**) ; 3) cells positive for both antibodies were seen (Fig. **1C** and **c**) ; 4) Cells negative for both antibodies are shown in (Fig. **1D** and **d**).

Relationship Between the Expression of CD44 and CD133 and Clinicopathological Factors

No significant association was found between CD44 and CD133 expression (P = 0.8535; Table **1**). In the CD44-positive group, there was a correlation between CD133 expression and clinicopathological factors, including lymph node metastasis (P = 0.0031) and lymphatic invasion (P = 0.0023) (Table **2**). In the CD44-negative group, however, no correlation between CD133 expression and clinicopathological factors was found (Table **2**).

Relationship Between Prognosis and Expression of CD44 and CD133

There was no significant difference in the 5-year survival rate of patients with tumors either positive (14.0%) or negative (6.5%) for CD44 expression (P = 0.1098; Fig. **2A**). The

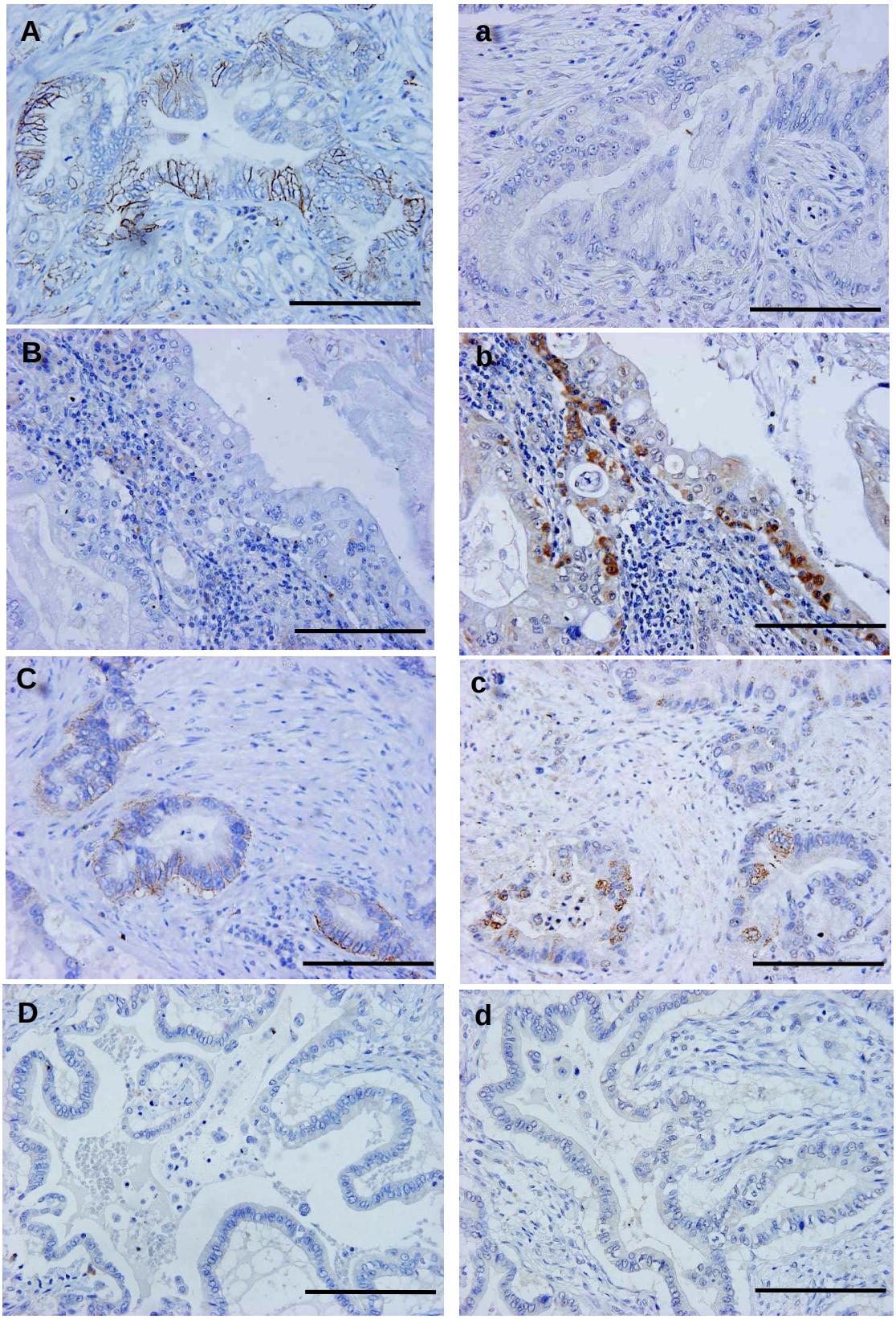

Fig. (1). Immunohistochemical staining for CD44 and CD133 in invasive ductal adenocarcinoma of the pancreas head. (**A**) CD44-positive area. CD44 expression appeared to be present on the cell surface of tumor cells. (**a**) Consecutive slices of (**A**) which are completely unreactive with anti-CD133 antibody. (**B**) CD44-negative area. (**b**) Consecutive slices of (**B**) which stained positively with anti-CD133 antibody. CD133 expression appeared to be present at the peripheral portions (facing the interstitial space) of the glandular structures of pancreatic adenocarcinoma, and stained intensely in the cytoplasm of tumor cells. (**C**) CD44-positive area. (**c**) Consecutive slices of (**C**) which stained positively with anti-CD133 antibody. (**D**) CD44-negative area. (**d**) Consecutive slices of (**D**) which are also unreactive with anti-CD133 antibody. Scale bars, 100 μm.

Table 1. Correlation Between Expression of CD44 and CD133 in Pancreatic Head Carcinoma

	CD44 Expression		*P*-Value
	Positive Expression (n = 34)	Negative Expression (n = 46)	
CD133 Expression			
Negative (n = 32)	14 (41.2%)	18 (39.1%)	0.8535
Positive (n = 48)	20 (58.8%)	28 (60.9%)	

5-year survival rate of patients with CD133-positive tumors was (2.1%), which was significantly different from that (23.5%) of patients with CD133-negative tumors (P = 0.0002; Fig. **2B**). In the CD44-positive group, the 5-year survival rate of the CD133-positive patients was significantly lower than that of the CD133-negative patients (P = 0.0010; Fig. **3A**). In the CD44-negative group, there was no significant difference between patients positive or negative for CD133 expression (P = 0.0743; Fig. **3B**).

Univariate and Multivariate Survival Analysis

Univariate analysis demonstrated that post-operative survival was significantly related to lymphatic invasion, lymph node metastasis, tumor depth, CD133 expression, and the combination of CD44 (+) expression and CD133 (-) expression ($P < 0.05$) (Table **3**). Multivariate regression analysis demonstrated that the CD44 (+) CD133 (-) status was an independent prognostic factor (P = 0.0424) (Table **3**).

Table 2. Relationship Between CD133 Expression and Clinicopathological Factors According to CD44 Expression

	CD44 Positive (n = 34)		*P* Value	CD44 Negative (n = 46)		*P* Value
	CD133 Positive n = 20 (58.8%)	CD133 Negative n = 14 (41.2%)		CD133 Positive n = 28 (60.9%)	CD133 Negative n = 18 (39.1%)	
Histology						
Well	7 (35.0)	10 (71.4)	0.0995	8 (28.6)	10 (55.6)	0.1522
Moderate	12 (60.0)	4 (28.6)		19 (67.9)	7 (38.9)	
Poor	1 (5.0)	0 (0.0)		1 (3.6)	1 (5.6)	
pT						
pT1	1 (5.0)	1 (7.1)	0.4084	0 (0.0)	1 (5.6)	0.3454
pT2	0 (0.0)	1 (7.1)		1 (3.6)	2 (11.1)	
pT3	17 (85.0)	12 (85.7)		24 (85.7)	12 (66.7)	
pT4	2 (10.0)	0 (0.0)		3 (10.7)	3 (16.7)	
pN						
Negative	3 (15.0)	9 (64.3)	0.0031	8 (28.6)	9 (50.0)	0.1417
Positive	17 (85.0)	5 (35.7)		20 (71.4)	9 (50.0)	
Liver Metastasis						
Negative	12 (60.0)	10 (71.4)	0.4925	16 (57.1)	13 (72.2)	0.3011
Positive	8 (40.0)	4 (28.6)		12 (42.9)	5 (27.8)	
pStage						
I	1 (5.0)	2 (14.3)	0.2268	1 (3.6)	2 (11.1)	0.7637
IIA	3 (15.0)	6 (42.9)		8 (28.6)	5 (27.8)	
IIB	14 (70.0)	6 (42.9)		15 (53.6)	7 (38.9)	
III	2 (10.0)	0 (0.0)		2 (7.1)	2 (11.1)	
IV	0 (0.0)	0 (0.0)		2 (7.1)	2 (11.1)	
Lymphatic Invasion						
Negative	1 (5.0)	7 (50.0)	0.0023	1 (3.6)	2 (11.1)	0.3121
Positive	19 (95.0)	7 (50.0)		27 (96.4)	16 (88.9)	
Venous Invasion						
Negative	4 (20.0)	4 (28.6)	0.5620	5 (17.9)	6 (33.3)	0.2298
Positive	16 (80.0)	10 (71.4)		23 (82.1)	12 (66.7)	

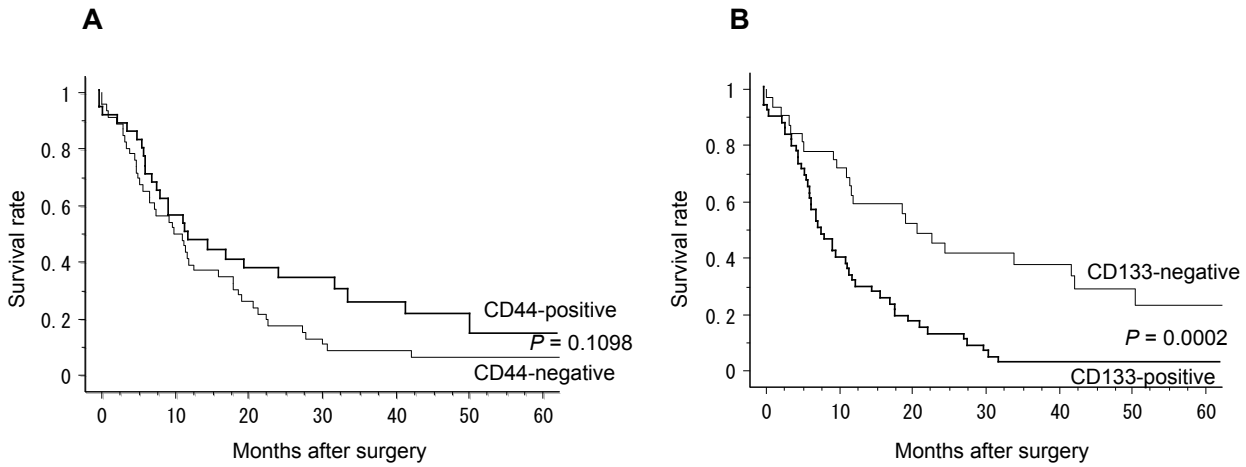

Fig. (2). (**A**) Kaplan-Meier survival curves for patients with CD44-positive and -negative in pancreatic head carcinomas. There is no significant difference between CD44-positive (14.0%) and -negative (6.5%) patients ($P = 0.1098$). (**B**) There is a significant difference in the 5-year survival rate between the CD133-positive (2.1%) and the CD133-negative patients (23.5%) ($P = 0.0002$).

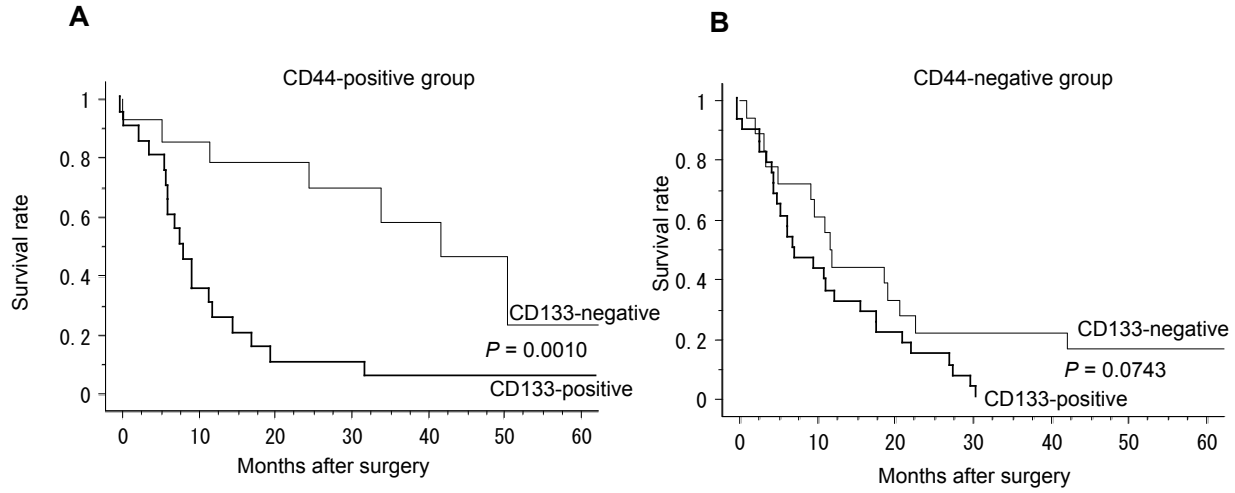

Fig. (3). Kaplan-Meier survival curves for the CD133-positive and -negative patients in the CD44-positive or negative group. (**A**) In the CD44-positive group, the 5-year survival rate was significantly lower for the CD133-positive patients than for the CD133-negative ones ($P = 0.0010$). (**B**) In the CD44-negative group, there is no significant difference between the CD133-positive and -negative patients ($P = 0.0743$).

Table 3. Univariate and Multivariate Analysis of Prognostic Factors in Pancreatic Head Carcinoma

Independent Factors	Univariate *P*	Multivariate *P*	Hazard Ratio	95% Confidence Interval
pT				
pT1, 2/pT3, 4	0.0417	0.2814	1.942	0.580-6.496
pN				
Negative/positive	0.0056	0.2232	1.459	0.794-2.681
Lymphatic Invasion				
Negative/positive	0.0334	0.6598	0.808	0.312-2.089
CD133 Expression				
Negative/positive	0.0002	0.3350	1.383	0.715-2.673
Combination of CD44 and CD133 Expression				
CD44 (+) CD133 (-)/Other groups	0.0009	0.0424	2.646	1.034-6.772

DISCUSSION

Stem cell-like tumor cells are thought to be tumor-initiating cells that are distinct from non-malignant stem cells and exhibit high rates of proliferation, a high capacity for self-renewal, and a propensity to differentiate into actively proliferating tumor cells [30-32]. In the present study, we employed immunohistochemical analyses for the detection of CSCs in the primary tumors of pancreatic ductal adenocarcinoma. First of all, the selection of appropriate antibodies was important. Concerning the CD133 antibody, at least three antibodies have been reported [33]. One antibody (clone AC133; Miltenyi Biotec, Bergisch Gladbach, Germany) was found to be reactive only with endoluminal cell membrane, but not with cell cytoplasm. We also observed clear reaction in the endoluminal cell membrane as well as in the cytoplasm of a small number of cells by another antibody (K-18; purchased from Santa Cruz) [24]. Therefore, we think that K-18 would be the most efficacious antibody for the recognition of CSCs. On the other hand, clone G44-26 (BD Pharmingen) for CD44 antibody is frequently used for detection of CSCs [34-36].

Many researchers have demonstrated that CSCs express CD44-related surface markers. A $CD44^+$ population of human head and neck squamous cell carcinoma cells was shown to give rise to new tumors *in vivo* [34], and $CD44^+$ expression was increased in tumorigenic and metastatic prostate cancer stem cells [37]. $CD44^+$ $CD24^-$ prostate cells have been shown to have tumorigenic potential and to correlate with poor survival [38]; additionally, $CD44^+$ $CD24^{-/low}$ epithelial-specific antigen $(ESA)^+$ cells [39], and $CD44^+$ $CD24^{-/low}$ cells [40] were able to drive breast cancer tumor formation *in vivo* and were associated with distant metastasis [41]. Pancreatic cancer cells with the $CD44^+$ $CD24^+$ ESA^+ phenotype were found to exhibit stem cell properties and an approximately 100-fold higher tumorigenic potential than non-tumorigenic cancer cells [42].

In this study, we assessed the possible expression of CD44 in pancreatic primary tumors. We observed the expression of CD44 in 42.5% (34 out of 80) of the evaluated tumors. However, no significant difference in the 5-year survival rate was noted between the CD44-positive and CD44-negative patients. As mentioned previously, in the case of tongue [9], laryngeal [10], and colorectal cancers [11], negative expression of CD44 is considered a poor prognostic factor. Although the reverse has been reported in colorectal cancer [12], most researchers agree that CD44 expression is not predictive of survival [43, 44]. We think that these facts would depend on the tumor types that were examined.

In the present study, there was no significant difference in the clinical outcome of patients with tumors positive or negative for CD44 expression. Therefore, we re-analyzed the data by dividing the patients into two groups based on their CD44 expression. In the CD44-positive group, CD133 expression correlated with lymph node metastasis and lymphatic invasion, and the 5-year survival rate was poorer in CD133-positive patients as compared to CD133-negative patients. On the other hand, in the CD44-negative group, no significant correlation was seen between CD133 expression and the clinicopathological factors, including prognosis. Univariate analysis revealed that the CD44 (+)/CD133 (-) patients experienced the best clinical outcome. Multivariate analysis also revealed that the CD44 (+)/CD133 (-) status is an independent prognostic factor. Taken together, it is conceivable that CD133 expression is involved in the appearance of CD44-positive tumors.

Recently, it was reported that the niche microenvironment of CSCs in brain tumors is important for maintenance of CSCs through close interaction with endothelial cells [45]. Unfortunately, it remains unclear at present whether CD44 expression would play roles in maintenance of the niche microenvironment where CSCs reside. We believe, however, that CD133 positive cells would exist under the niche microenvironment of CSCs in pancreatic cancer.

In conclusion, our immunohistochemical results indicate that the combination of CD44 and CD133 expression is a useful marker for the prediction of clinical outcome in pancreatic cancer patients. Although CD44 has been considered to play a role in adhesion to target organs during the metastatic process, another role such as prevention of the release of CSCs from the niche should be investigated.

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