CD133 expression predicts post-operative recurrence in patients with colon cancer with peritoneal metastasis

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Abstract. Despite extensive research on cancer stem cells in colorectal cancer, the impact of stem cell markers on patient survival remains unclear, particularly in those with distant metastasis. In this study, we focused on colon cancer with peritoneal metastasis and investigated the association between the expression of CD133, aldehyde dehydrogenase-1 (ALDH1) and leucine-rich repeating G-protein coupled receptor-5 (Lgr5), and disease prognosis. Putative stem cell marker expression was immunohistochemically evaluated in samples from 142 primary tumours and 75 peritoneal nodules. The associations between the expression of these markers and clinicopathological characteristics, overall survival and disease-free survival were analysed. The expression of CD133, ALDH1 and Lgr5 was found to be positive in 55.6, 47.2 and 78.9% of the primary tumour samples, respectively. While their expression was not associated with overall survival, disease-free survival was significantly worse in the CD133-negative group (36.1 vs. 13.7%, P=0.041). Multivariable analysis confirmed that a negative CD133 expression was an independent risk factor for a reduced disease-free survival (P=0.005). Furthermore, the benefit of systemic chemotherapy was significantly greater in the CD133-negative group (P=0.039). On the whole, our data indicated that patients with colon cancer with CD133-negative expression had a reduced disease-free survival. Thus, we propose that CD133 expression may be a useful clinical biomarker in the treatment of colon cancer with peritoneal metastasis.

Introduction

Distant metastasis, a major prognosis determinant in patients with colorectal cancer, is present in approximately one-fifth of all newly diagnosed cases. The 5-year survival of patients who present with synchronous distant metastases is not >20% (1,2). Moreover, even if patients are initially diagnosed with non-metastatic disease, 25-50% of these patients experience tumour recurrence despite potentially curative resections (1,3,4). In order to improve the prognoses of these patients, it is essential to investigate the aetiology and pathophysiology of metastasis and find an innovative therapeutic strategy.

It has recently been proposed that cancer stem cells play a significant role in tumour progression and metastatic dissemination in various types of cancer (5). A cancer stem cell, defined as 'a cell within a tumour that possesses the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumour' (6), is considered to be an explanation for the resistance of cancer tissues to treatment. Therefore, tumours with high expression levels of cancer stem cell markers are expected to exhibit unfavourable prognoses.

However, the impact of cancer stem cell markers on patient survival remains unclear, particularly in patients with distant metastasis. Our previous study on liver metastasis using CD133, the most well known putative stem cell marker in colorectal cancer, revealed that patients without CD133 expression exhibited reduced survival (7). Although this seemed to contradict the cancer stem cell theory, there are other studies which support these findings (8,9).

Therefore, in this study, we focused on patients with colon cancer with peritoneal metastasis, as this has not been investigated to date, at least to the best of our knowledge. The aim of this study was to determine the impact of cancer stem cell markers on the prognosis of patients with colon cancer with peritoneal metastasis. We also examined 2 other putative stem cell markers, aldehyde dehydrogenase-1 (ALDH1) and

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leucine-rich repeating G-protein coupled receptor-5 (Lgr5), in order to examined whether the findings may be attributed to stemness or whether they are specific to CD133.

Materials and methods

Patients and tissue specimens. Among 1,990 patients who underwent resection for primary sporadic colon cancer at the University of Tokyo Hospital (Tokyo, Japan) between 1997 and 2015, 171 were diagnosed with synchronous or metachronous peritoneal metastasis. Information on these patients and their tumour characteristics, treatments and clinical outcomes were retrieved from patient medical records. During the former half of the study period, the majority of surgeries were performed by an open method, while in the latter half, approximately one third of the surgeries were performed laparoscopically. A total of 55 patients underwent complete resections of recognisable metastases, including visible peritoneal nodules, while the remaining patients had residual tumour tissues. No patients underwent peritonectomy or hyperthermic intraperitoneal chemotherapy (HIPEC).

The study protocol was approved by the Research Ethics Committee at the Graduate School of Medicine, the University of Tokyo (Tokyo, Japan) (approval no. G3552-3). All participants provided informed, written informed consent. This study was conducted in accordance with the 1964 Declaration of Helsinki and its later amendments.

Peri-operative evaluation. All patients were clinically staged by a physical examination, colonoscopy and chest-abdomen-pelvis computed tomography (CT) prior to surgery. Pathological staging was performed according to the Union for International Cancer Control TNM Classification of Malignant Tumours, 7th edition (10). Tumours proximal to the splenic flexure were classified as proximal colon cancer and those distal to the splenic flexure were classified as distal colon cancer. Tumours originating in the vermiform appendix and rectum were excluded. In all cases, regular follow-up examinations were performed (tumour marker assessments every 3 months, chest-abdomen-pelvic CT every 6 months and an annual total colonoscopy). Peritoneal metastasis was diagnosed based on surgical exploration or imaging studies. No patient underwent systematic second-look surgery.

Evaluation by immunohistochemistry. Consecutive 3-μm-thick formalin-fixed paraffin-embedded sections were immunohistochemically stained manually using the following procedures. Following deparaffinisation and rehydration, endogenous peroxidase was blocked with 3% hydrogen peroxidase solution in methanol for 15 min. Heat-induced antigen retrieval was performed in 10 mM sodium citrate buffer (pH 6.0) using an autoclave. Following non-specific protein blocking by incubation with 5% bovine serum albumin for 30 min, the slides were incubated overnight with primary antibodies against CD133 (mouse anti-human polyclonal antibody; AC133; 1:100 dilution; Miltenyi Biotec, Auburn, CA, USA), ALDH1 (clone EP1933Y; 1:200 dilution; Abcam, Cambridge, MA, USA) and Lgr5 (clone EPR3065Y; 1:100 dilution; LifeSpan Biosciences, Seattle, WA, USA), in a humidified container at 4˚C. They were then incubated using a Dako Envision kit (Dako, Carpinteria,

CA, USA) and also incubated in 2% 3,3'-diaminobenzidine tetrahydrochloride and 50 mM tris-buffer containing hydrogen peroxidase as a chromogen following the manufacturer's instructions. Meyer's haematoxylin (Sigma Chemical Co., St. Louis, MO, USA) was used for counterstaining.

Expression was defined as positive when CD133, ALDH1 and Lgr5 staining was found in >5, 20 and 50% of the tumour samples, respectively (7,11,12), by examining 1,000 tumour cells in 10 fields (100 cells/field) with high-power (x200) microscopy (11). The evaluation was performed independently by 2 clinicians (H.N. and J.K.) who were blinded to the clinical findings. Discrepancies between their findings were resolved by discussion.

Statistical analysis. Categorical variables were described using frequencies and percentages. Correlation was evaluated using Fisher's exact test or a Chi-squared test. The distributions of continuous variables were described using medians and the interquartile ranges. The Mann-Whitney test was used for comparisons. Overall survival was defined as the duration between the date of primary tumour resection and the date of death from any cause. Disease-free survival was defined as the duration between the date of complete resection for metastasis and the date of recurrence or death from any cause. These outcomes were calculated using the Kaplan-Meier method and compared using the log-rank test. Univariate and multivariate Cox regression analyses were performed to investigate patient and tumour characteristics associated with the prognosis of metachronous peritoneal metastasis. The variables for multivariate analysis were selected using the model selection approach by Collett (13). Through the time-to-event analysis, hazard ratios (HRs) and 95% confidence intervals (CIs) were generated. Associations were considered significant for P-values <0.05 in general; however, a level of significance of 0.15 was used for Collett's univariable screening (13). Data were statistically analysed using the statistical program R version 3.3.1 (http://www.R-project.org/).

Results

Immunohistochemical expression in primary tumours and clinicopathological variables. Specimens of 142 cases were available for the immunohistological evaluation of primary tumours, and those of 75 cases were available for the evaluation of metastatic peritoneal nodules, as shown in Fig. 1. The immunohistochemical staining pattern of CD133, ALDH1, and Lgr5 is shown in Fig. 2. Membranous immunoreactivity was observed for CD133, and cytoplasmic immunoreactivity was observed for ALDH1 and Lgr5. The rates of the positive expression of CD133, ALDH1 and Lgr5 in our primary tumour samples were 55.6% (79/142), 47.2% (67/142) and 78.9% (112/142), respectively.

The clinicopathological characteristics of the patients are shown in Table I. Concurrent distant metastasis was found in 59 patients. While 33 of these patients had a single organ metastasis other than the peritoneum, 20 patients had 2, and 6 patients had more than 3 sites of metastasis in addition to the peritoneum. The most common site of metastasis was the liver (35 patients), followed by the lung (20 patients), the distant lymph nodes (19 patients) and the ovary (6 patients).
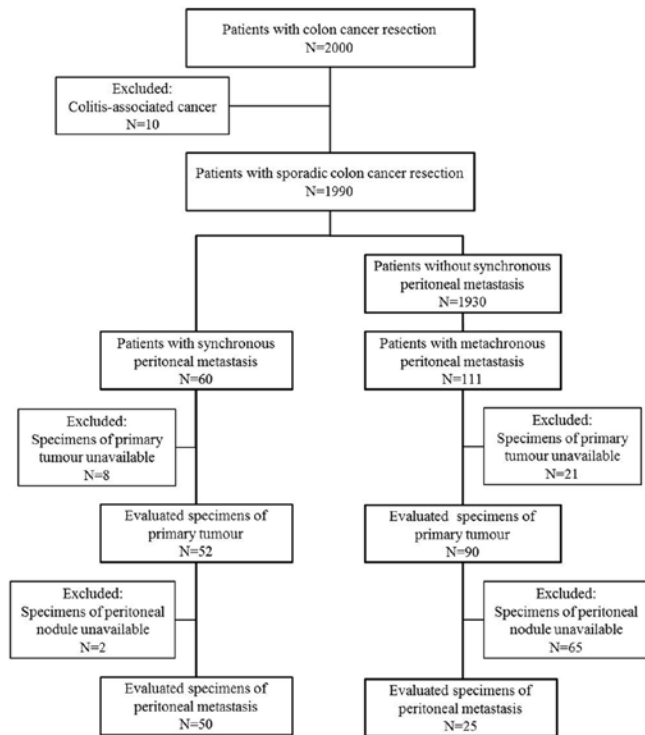
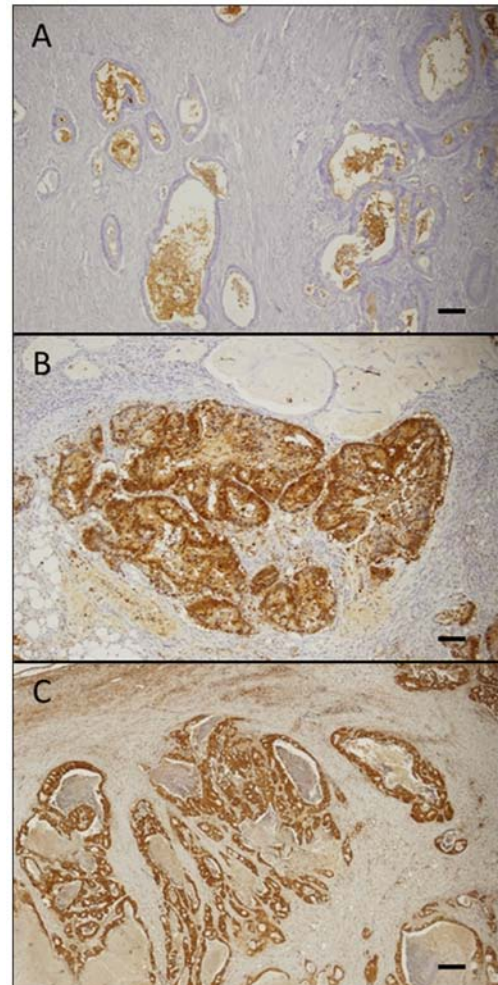Figure 1. Flow chart for the selection of specimens.

Figure 2. Expression of cancer stem cell markers by immunohistochemical staining. (A) CD133, (B) aldehyde dehydrogenase-1 (ALDH1) and (C) leucine-rich repeating G-protein coupled receptor-5 (Lgr5) (original magnification, x200; scale bars, 100 μm).

With regards to adjuvant chemotherapy following the resection of the peritoneal nodules, 5-fluorouracil regimens were used in 16 patients and cytotoxic doublet regimens were used in 20 patients.

The expression levels of CD133 and Lgr5 were significantly lower in the mucinous adenocarcinoma samples (P<0.001 and P=0.020). In addition, the expression of CD133 significantly correlated with differentiation (P<0.001) and the N category (P=0.002), and the percentage of complete resection was significantly lower in the Lgr5-negative group (P=0.002) (Table I).

Immunohistochemical expression in primary tumours and prognosis. The median overall survival of the patients with peritoneal metastasis was 24.6 months, and the 5-year overall survival rate was 22.0% among all patients. The expression of CD133, ALDH1 and Lgr5 was not associated with overall survival (29.7 vs. 12.7%, P=0.158 for CD133; 20.2 vs. 23.8%, P=0.553 for ALDH1; and 19.4 vs. 31.1%, P=0.086 for Lgr5) (Fig. 3A-C). Similar results were observed when the patient subgroups were stratified according to the completeness of metastasectomy (P=0.123, P=0.265 and P=0.619 for CD133, ALDH1 and Lgr5, respectively, in the patients with complete resection, and P=0.956, P=0.993 and P=0.718 for CD133, ALDH1 and Lgr5, respectively, in the patients with residual metastasis) (Fig. 4).

The median disease-free survival of 55 patients who underwent complete resection was 16.0 months and the 5-year disease-free survival rate for these patients was 27.2%. The most common site of recurrence was the lung (5 patients), followed by the peritoneum, liver and distant lymph nodes (4 patients for each). While no significant difference was observed in the expression of ALDH1 and Lgr5 (31.2 vs. 24.1%,

P=0.433 for ALDH1 and 23.6 vs. 33.3%, P=0.236 for Lgr5), a positive expression of CD133 was associated with a significantly improved 5-year disease-free survival (36.1 vs. 13.7%, P=0.041) (Fig. 3D-F).

The Cox multivariate model selected using the Collett's model selection approach contained age, adjuvant chemotherapy following peritoneal nodule resection, and the expression of CD133. A positive CD133 expression was found to be a significant positive factor for disease-free survival (HR, 0.33; 95% CI, 0.16-0.72, P=0.005) as was adjuvant chemotherapy (HR, 0.23; 95% CI, 0.10-0.52, P<0.001) and older age (HR, 0.24; 95% CI, 0.08-0.63 for patients aged 50-69 years; and HR, 0.16, 95% CI, 0.05-0.48 for patients aged ≥70 years, P=0.003) (Table II).

Immunohistochemical expression in peritoneal nodules and clinicopathological variables. The rate of the positive expression of CD133, ALDH1 and Lgr5 was 40.0% (30/75), 30.7% (23/75) and 78.6% (59/75), respectively, in our peritoneal nodule samples. The expression of CD133 and ALDH1 in the peritoneal nodules was significantly lower than that in the primary tumour samples (P=0.002 and P=0.001, respectively), while that of Lgr5 did not differ significantly (P=0.207).

Table I. Clinicopathological characteristics of the patients from whom primary tumour samples were obtained.

	CD133			ALDH1			Lgr5		
	(+) n=79 n (%)	(-) n=63 n (%)	P-value	(+) n=67 n (%)	(-) n=75 n (%)	P-value	(+) n=112 n (%)	(-) n=30 n (%)	P-value
Age, years			0.701			0.218			0.757
Median	64	64		65	25		65	64	
(interquartile range)	(55-75)	(58-74)		(57-77)	(55-73)		(55-75)	(58-72)	
Sex			0.983			0.545			0.963
Male	45 (57.0)	36 (57.1)		40 (59.7)	41 (54.7)		64 (57.1)	17 (56.7)	
Female	34 (43.0)	27 (42.9)		27 (40.3)	34 (45.3)		48 (42.9)	13 (43.3)	
Site of primary tumour			0.707			0.119			0.056
Proximal (right)	30 (38.0)	22 (34.9)		29 (43.3)	23 (30.7)		46 (41.1)	6 (20.0)	
Distal (left)	49 (62.0)	41 (65.1)		38 (56.7)	52 (69.3)		66 (59.9)	24 (80.0)	
Differentiation			<0.001[a]			0.078			0.340
Well	32 (40.5)	25 (39.7)		22 (32.9)	35 (46.7)		45 (40.2)	12 (40.0)	
Moderate	47 (59.5)	24 (38.1)		35 (52.2)	36 (52.0)		58 (51.8)	13 (33.3)	
Poor	0 (0.0)	14 (22.2)		10 (14.9)	4 (5.3)		9 (8.0)	5 (16.7)	
Histology			<0.001[a]			0.067			0.020[a]
Tubular adenocarcinoma	78 (98.7)	52 (82.5)		58 (86.6)	72 (96.0)		106 (94.6)	24 (80.0)	
Mucinous adenocarcinoma	1 (1.3)	11 (17.5)		9 (13.4)	3 (4.0)		6 (5.4)	6 (20.0)	
T category			0.582			0.200			0.991
T1-3	23 (29.1)	16 (25.4)		22 (32.8)	17 (22.7)		31 (27.7)	8 (26.6)	
T4a	41 (51.9)	38 (60.3)		32 (47.8)	47 (62.7)		62 (55.4)	17 (56.7)	
T4b	15 (19.0)	9 (14.3)		13 (19.4)	11 (14.6)		19 (17.0)	5 (16.7)	
N category			0.002[a]			0.484			0.631
N0	31 (39.2)	12 (19.1)		21 (31.3)	22 (29.3)		36 (32.1)	7 (23.4)	
N1	32 (40.5)	22 (34.9)		28 (41.8)	26 (34.7)		41 (36.6)	13 (43.3)	
N2	16 (20.3)	29 (46.0)		18 (26.9)	27 (36.0)		35 (31.3)	10 (33.3)	
Examined lymph node count			0.628			0.717			0.708
<12	19 (24.1)	13 (20.6)		16 (23.9)	16 (21.3)		26 (23.2)	6 (20.0)	
≥12	60 (75.9)	50 (79.4)		51 (76.1)	59 (78.7)		86 (76.8)	24 (80.0)	
Lymphatic invasion			0.416			0.686			0.830
ly0	38 (48.1)	26 (41.3)		29 (43.3)	35 (46.7)		51 (12.2)	13 (33.3)	
ly1	41 (51.9)	37 (58.7)		38 (56.7)	40 (53.3)		61 (54.5)	17 (56.7)	
Venous invasion			0.200			0.946			0.080
v0	10 (12.7)	13 (20.6)		11 (16.4)	12 (16.0)		15 (13.4)	8 (26.6)	
v1	69 (87.3)	50 (79.4)		56 (83.6)	63 (84.0)		97 (86.6)	22 (73.4)	
Concurrent metastasis			0.276			0.531			0.148
Peritoneum alone	43 (54.4)	40 (63.5)		41 (61.2)	42 (56.0)		62 (55.4)	21 (70.0)	
Other metastasis	36 (45.6)	23 (36.5)		26 (38.8)	33 (44.0)		50 (44.6)	9 (30.0)	
1 Other metastatic site	17 (21.5)	16 (25.4)		13 (19.4)	20 (26.7)		27 (24.1)	6 (20.0)	
2 Other metastatic sites	15 (19.0)	5 (7.9)		10 (14.9)	10 (13.3)		17 (15.2)	3 (10.0)	
≥3 Metastatic sites	4 (5.1)	2 (3.2)		3 (4.5)	3 (4.0)		6 (5.4)	0 (0.0)	
Presentation			0.468			0.057			0.674
Synchronous metastasis	31 (39.2)	21 (33.3)		30 (44.8)	22 (29.3)		42 (37.5)	10 (33.3)	
Metachronous metastasis	48 (60.8)	42 (66.7)		37 (55.2)	53 (70.7)		70 (62.5)	20 (66.7)	
Peritoneal cancer index			0.156			0.470			0.157
<10	65 (82.3)	47 (74.6)		50 (74.6)	62 (82.7)		86 (76.8)	26 (86.6)	
10-20	13 (16.4)	11 (17.5)		13 (19.4)	11 (14.6)		22 (19.6)	2 (6.7)	
>20	1 (1.3)	5 (7.9)		4 (6.0)	2 (2.7)		4 (35.7)	2 (6.7)	
Status of metastasectomy			0.627			0.717			0.002
Complete resection	32 (40.5)	23 (36.5)		27 (40.3)	28 (37.3)		36 (32.1)	19 (63.3)	
Residual metastasis (+)	47 (59.5)	40 (63.5)		40 (59.7)	47 (62.7)		76 (67.9)	11 (36.7)	

Table I. Continued.

	CD133			ALDH1			Lgr5		
	(+) n=79 n (%)	(-) n=63 n (%)	P-value	(+) n=67 n (%)	(-) n=75 n (%)	P-value	(+) n=112 n (%)	(-) n=30 n (%)	P-value
Chemotherapy after metastasectomy			0.544			0.853			0.795
Adjuvant chemotherapy (-)	10 (12.7)	9 (14.3)		9 (13.4)	10 (13.3)		12 (10.7)	7 (23.4)	
Adjuvant chemotherapy (+)	22 (27.8)	14 (22.2)		18 (26.9)	18 (24.0)		24 (21.4)	12 (40.0)	
[b]5-FU	12 (15.1)	4 (6.3)		4 (6.0)	12 (16.0)		11 (9.8)	5 (16.7)	
[c]FOLFOX/[d]CapeOX	10 (12.7)	10 (15.9)		14 (20.9)	6 (8.0)		13 (11.6)	7 (23.3)	

[a]Indicates statistical significance (P<0.05); [b]5-fluorouracil; [c]folinic acid, 5-fluorouracil and oxaliplatin; [d]capecitabine and oxaliplatin.
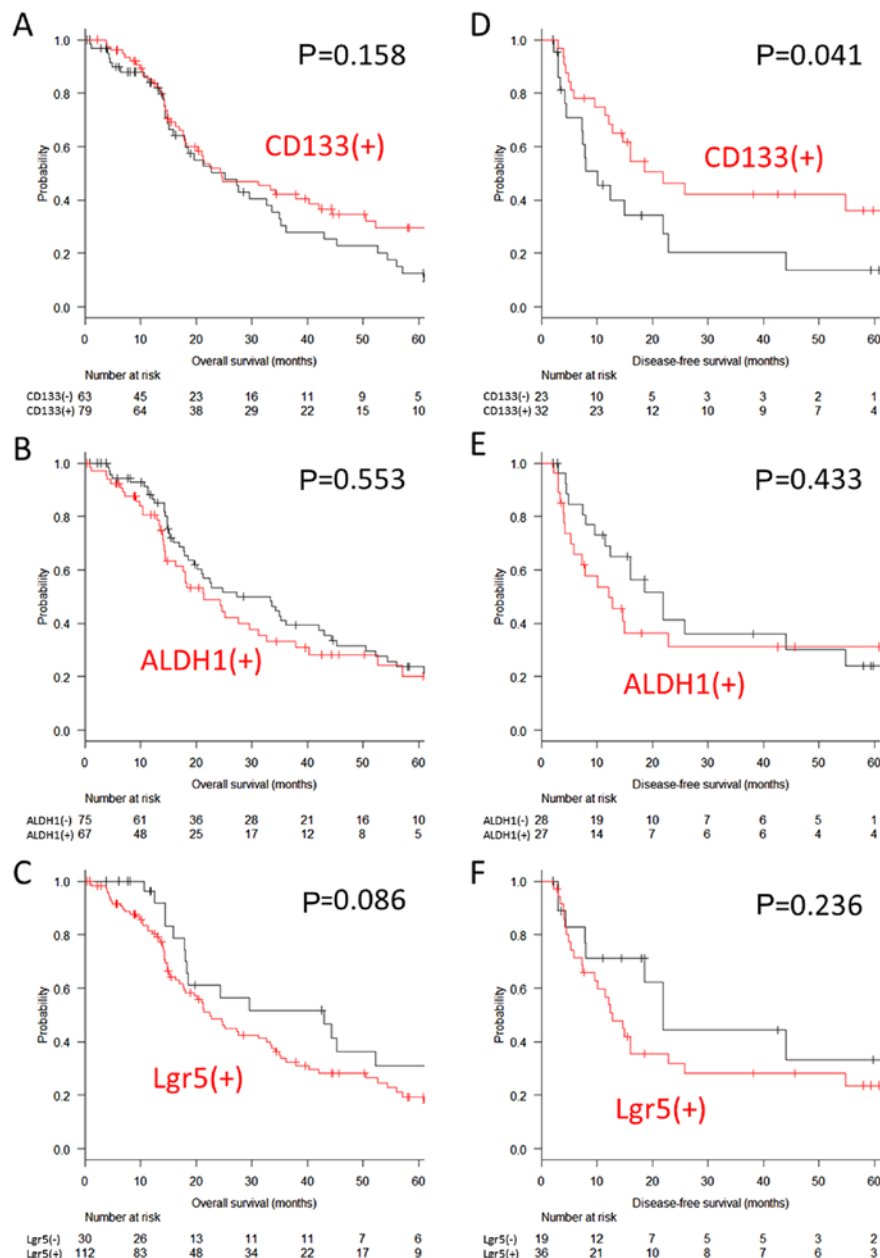

Figure 3. Association of stem cell marker expression levels in primary tumours with prognosis. Overall survival according to the expression of (A) CD133, (B) aldehyde dehydrogenase-1 (ALDH1), and (C) leucine-rich repeating G-protein coupled receptor-5 (Lgr5). Disease-free survival according to the expression of (D) CD133, (E) ALDH1, and (F) Lgr5.

Table II. Association between clinicopathological variables and disease-free survival.

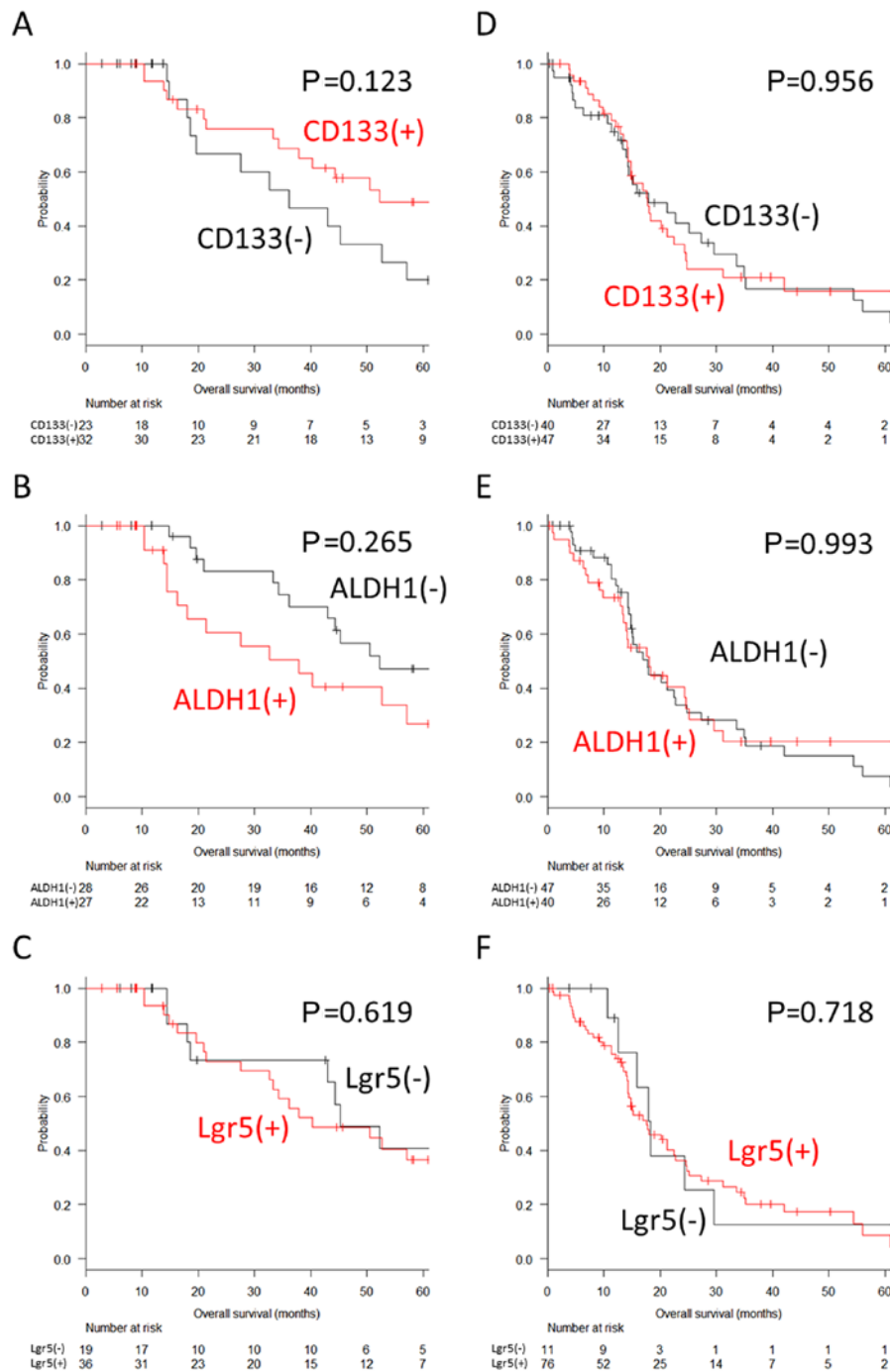|	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
Age, years			0.174			0.003
<50	Reference			Reference		
50-69	0.45	0.17-1.16		0.23	0.09-0.63	
≥70	0.39	0.13-1.13		0.16	0.05-0.48	
Sex			0.462			
Male	Reference					
Female	1.30	0.65-2.60				
Site of primary tumour			0.254			
Proximal (right)	Reference					
Distal (left)	0.66	0.32-1.35				
Differentiation			0.086			
Well	Reference					
Moderate	1.54	0.74-3.18				
Poor	3.55	1.14-11.05				
Histology			0.465			
Tubular adenocarcinoma	Reference					
Mucinous adenocarcinoma	1.56	0.47-5.14				
T category			0.937			
T3	Reference					
T4a	1.08	0.49-2.35				
T4b	0.90	0.27-2.91				
N category			0.281			
N0	Reference					
N1	1.41	0.54-3.70				
N2	2.10	0.80-5.48				
No. of examined lymph nodes			0.555			
<12	Reference					
≥12	0.77	0.32-1.86				
Lymphatic invasion			0.291			
ly0	Reference					
ly1	1.45	0.73-2.88				
Venous invasion			0.881			
v0	Reference					
v1	0.92	0.32-2.63				
Concurrent metastasis			0.548			
Peritoneum alone	Reference					
Other metastasis	0.75	0.29-1.93				
Presentation of peritoneal metastasis			0.599			
Synchronous	Reference					
Metachronous	0.83	0.42-1.64				
Adjuvant chemotherapy			0.012[a]			<0.001[a]
Adjuvant (-)	Reference			Reference		
Adjuvant (+)	0.40	0.20-0.82		0.23	0.10-0.52	
CD133 expression			0.045[a]			0.005[a]
CD133(-)	Reference			Reference		
CD133(+)	0.50	0.25-0.99		0.33	0.16-0.72	

[a]Indicates statistical significance (P<0.05).

Figure 4. Association of stem cell marker expression levels in primary tumours with overall survival by the completeness of metastasectomy. Overall survival of patients with complete resection of metastasis according to the expression of (A) CD133, (B) aldehyde dehydrogenase-1 (ALDH1), and (C) leucine-rich repeating G-protein coupled receptor-5 (Lgr5). Overall survival of patients with residual metastasis according to the expression of (D) CD133, (E) ALDH1 and (F) Lgr5.

The clinicopathological characteristics of the patients with peritoneal metastasis are shown in Table III. CD133-negative peritoneal nodules significantly correlated with differentiation (P=0.0030), but not with mucinous adenocarcinoma (P=0.392) or the N category (P=0.119). The number of patients with <12 examined lymph nodes was higher in those with ALDH1-negative peritoneal nodules (P=0.016).

Immunohistochemical expression in peritoneal nodules and prognosis. The impact of immunohistochemical expression in peritoneal nodules on prognosis was similar to that of the primary tumour. The expression of CD133, ALDH1 and Lgr5 was not associated with overall survival (46.4 vs. 20.9%, P=0.617 for CD133; 35.7 vs. 26.9%, P=0.576 for ALDH1; and 29.0 vs. 34.8%, P=0.679 for Lgr5) (Fig. 5A-C). By contrast, the 5-year disease-free survival after complete surgery was significantly superior in the patients with CD133-positive peritoneal nodules (39.5 vs. 22.4%, P=0.040). However, no significant difference was observed in the expression of ALDH1 and Lgr5 in peritoneal nodules (27.9 vs. 34.7%, P=0.906 for ALDH1; and 27.5 vs. 45.8%, P=0.767 for Lgr5) (Fig. 5D-F).

Table III. Clinicopathological characteristics of the patients with peritoneal nodules.

	CD133			ALDH1			Lgr5		
	(+) n=30 n (%)	(-) n=45 n (%)	P-value	(+) n=23 n (%)	(-) n=52 n (%)	P-value	(+) n=59 n (%)	(-) n=16 n (%)	P-value
Age, years			0.095			0.936			0.969
Median	68	61		62	65		64	64	
(interquartile range)	(60-72)	(53-70)		(58-71)	(55-71)		(57-71)	(55-75)	
Sex			0.850			0.773			0.886
Male	16 (53.3)	25 (55.6)		12 (52.2)	29 (55.8)		32 (54.2)	9 (56.3)	
Female	14 (46.7)	20 (44.4)		11 (47.8)	23 (44.2)		27 (45.8)	7 (43.7)	
Site of primary tumour			0.104			0.205			0.726
Proximal (right)	9 (30.0)	22 (48.9)		12 (52.2)	19 (36.5)		25 (42.4)	6 (37.5)	
Distal (left)	21 (70.0)	23 (51.1)		11 (47.8)	33 (63.5)		34 (57.6)	10 (62.5)	
Differentiation			0.030[a]			0.719			0.177
Well	12 (40.0)	18 (40.0)		8 (34.8)	22 (42.3)		22 (37.3)	8 (50.0)	
Moderate	18 (60.0)	19 (42.2)		13 (56.5)	24 (46.2)		32 (54.2)	5 (31.2)	
Poor	0 (0.0)	8 (17.8)		2 (8.7)	6 (11.5)		5 (8.5)	3 (18.8)	
Histology			0.392			1.000			0.602
Tubular adenocarcinoma	29 (96.7)	40 (88.9)		21 (91.3)	48 (92.3)		55 (93.2)	14 (88.5)	
Mucinous adenocarcinoma	1 (3.3)	5 (11.1)		2 (8.7)	4 (7.7)		4 (6.8)	2 (12.5)	
T category			0.625			0.449			0.523
T1-3	9 (30.0)	10 (22.2)		8 (34.8)	11 (21.1)		15 (25.4)	4 (25.0)	
T4a	16 (53.3)	24 (53.3)		11 (47.8)	29 (55.8)		33 (55.9)	7 (43.7)	
T4b	5 (16.7)	11 (24.4)		4 (17.4)	12 (23.1)		11 (18.6)	5 (31.3)	
N category			0.119			0.734			0.636
N0	11 (36.6)	10 (22.2)		8 (34.8)	13 (25.0)		18 (30.5)	3 (18.8)	
N1	14 (46.7)	18 (40.0)		9 (39.1)	23 (44.2)		25 (42.4)	7 (43.7)	
N2	5 (16.7)	17 (37.8)		6 (26.1)	16 (30.8)		16 (27.1)	6 (37.5)	
Examined lymph count			0.828			0.016[a]			1.000
<12	8 (26.7)	11 (24.4)		10 (43.5)	9 (17.3)		15 (25.4)	4 (25.0)	
≥12	22 (73.3)	34 (75.6)		13 (56.5)	43 (82.7)		44 (74.6)	12 (75.0)	
Lymphatic invasion			0.336			0.540			0.135
ly0	14 (46.7)	16 (35.6)		8 (34.8)	22 (42.3)		21 (35.6)	9 (56.3)	
ly1	16 (53.3)	29 (64.4)		15 (65.2)	30 (57.7)		38 (64.4)	7 (43.7)	
Venous invasion			0.746			1.000			0.692
v0	5 (16.7)	6 (13.3)		3 (13.0)	8 (15.4)		8 (13.6)	3 (18.8)	
v1	25 (83.3)	39 (86.7)		20 (87.0)	44 (84.6)		51 (86.4)	13 (81.2)	
Concurrent metastasis			0.840			0.465			0.242
Peritoneum alone	20 (66.7)	31 (68.9)		17 (74.0)	34 (65.4)		38 (64.4)	13 (81.2)	
Other metastasis	10 (33.3)	14 (31.1)		6 (26.0)	18 (34.6)		21 (35.6)	3 (18.8)	
Presentation			0.134			0.215			0.842
Synchronous metastasis	17 (56.7)	33 (73.3)		13 (56.5)	37 (71.2)		39 (66.1)	11 (68.7)	
Metachronous metastasis	13 (43.3)	12 (26.7)		10 (43.5)	15 (28.8)		20 (33.9)	5 (31.3)	
Peritoneal cancer index			0.511			0.263			0.788
<10	24 (80.0)	31 (68.9)		17 (74.0)	38 (73.1)		42 (71.2)	13 (81.3)	
10-20	4 (13.3)	11 (24.4)		3 (13.0)	12 (23.1)		13 (22.0)	2 (12.5)	
>20	2 (6.7)	3 (6.7)		3 (13.0)	2 (3.8)		4 (6.8)	1 (6.2)	
Status of metastasectomy			0.419			0.465			0.499
Complete resection	22 (73.3)	29 (64.4)		17 (74.0)	34 (65.4)		39 (66.1)	12 (75.0)	
Residual metastasis (+)	8 (26.7)	16 (35.6)		6 (26.0)	18 (34.6)		20 (33.9)	4 (25.0)	
Chemotherapy after metastasectomy			0.689			0.358			1.000
Adjuvant chemotherapy (-)	8 (26.7)	9 (20.0)		4 (17.4)	13 (25.0)		13 (22.0)	4 (25.0)	
Adjuvant chemotherapy (+)	14 (46.7)	20 (44.4)		13 (56.5)	21 (40.4)		26 (44.1)	8 (50.0)	

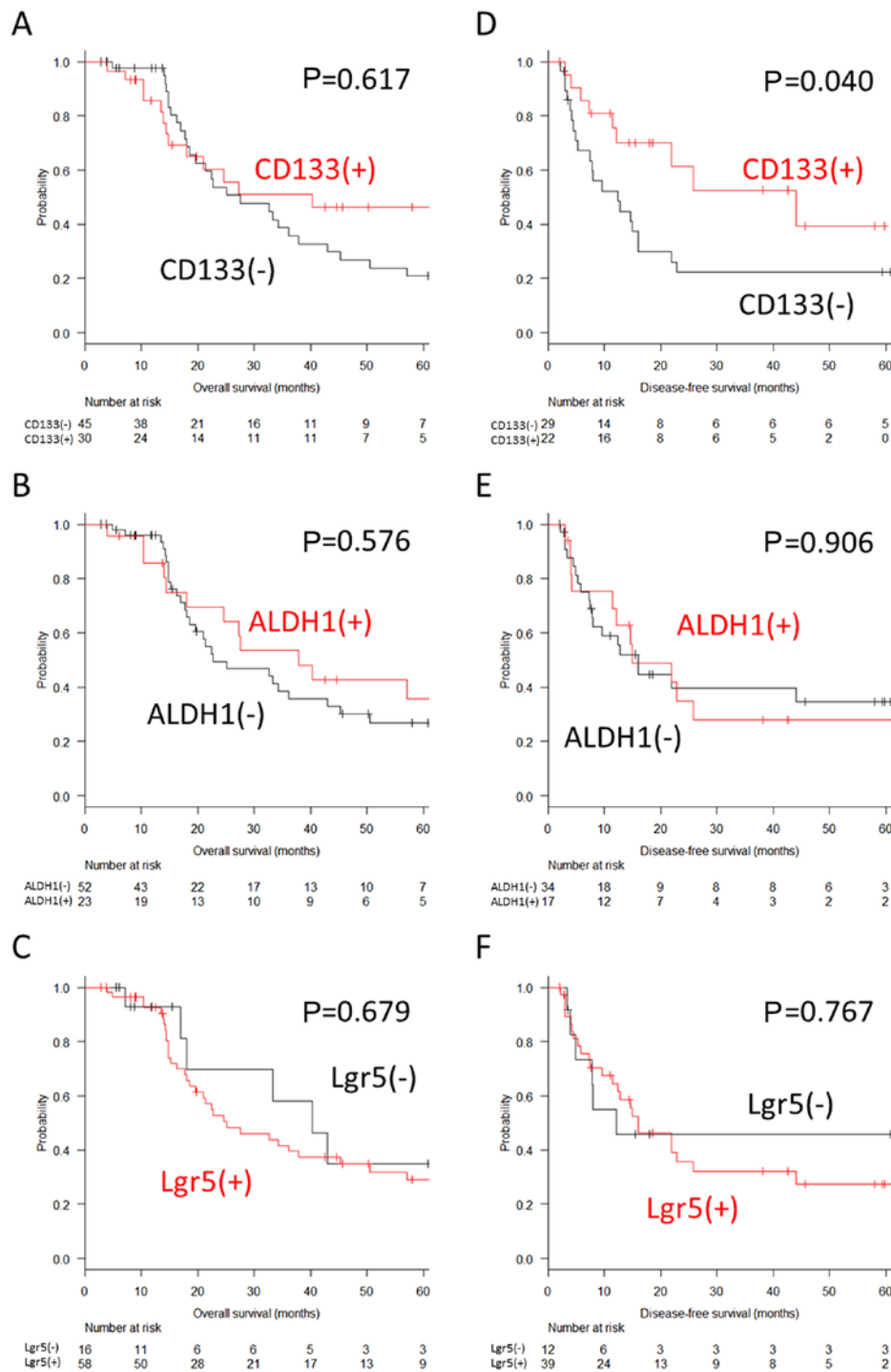[a]Indicates statistical significance (P<0.05).

Figure 5. Association of stem cell marker expression levels in peritoneal nodules with prognosis. Overall survival according to the expression of (A) CD133, (B) aldehyde dehydrogenase-1 (ALDH1), and (C) leucine-rich repeating G-protein coupled receptor-5 (Lgr5). Disease-free survival according to the expression of (D) CD133, (E) ALDH1, and (F) Lgr5.

The CD133 expression patterns were clearly associated with disease-free survival. The optimal survival was observed when both the primary tumour and peritoneal nodules were CD133-positive (double positive, Fig. 6), and this was followed by the survival of patients with CD133 positivity either in the primary tumour or peritoneal nodules (single positive). The worst survival was observed when CD133 expression was negative in both the primary tumour and peritoneal nodule (double negative) (49.8, 33.0 and 13.7%, respectively; P-value for the trend, P=0.011) (Fig. 7).

CD133 expression and chemosensitivity. We further evaluated the sensitivity to chemotherapy according to CD133 expression. Systemic chemotherapy significantly improved the overall survival of the CD133-negative group (HR, 0.19; 95% CI, 0.07-0.50, P<0.001) while the risk reduction in the CD133-positive group was not statistically significant (HR, 0.61; 95% CI, 0.27-1.38, P=0.231). Furthermore, the positive impact of systemic chemotherapy in the CD133-negative group was significantly greater than that in the CD133-positive group (P-value for the interaction, P=0.039).
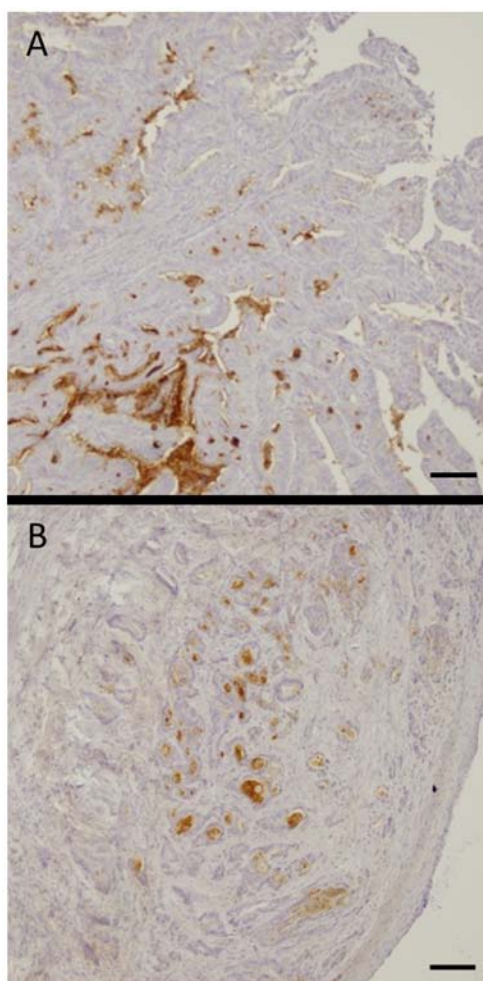
Figure 6. CD133 staining pattern in (A) a primary tumour and (B) a peritoneal nodule (original magnification, x100; scale bars, 100 μm).

With regard to adjuvant chemotherapy following resection, the risk reduction in disease-free survival was statistically significant only in the CD133-negative group (HR, 0.48; 95% CI, 0.18-1.25, P=0.132 in the CD133-positive group; and HR, 0.24; 95% CI, 0.07-0.80, P=0.021 in the CD133-negative group). However, the impact was not statistically significant between the 2 groups (P-value for the interaction, P=0.345).

Discussion

In this study, we investigated the expression of 3 cancer stem cell markers in patients with colon cancer with peritoneal metastasis, and revealed that CD133-negative patients had a reduced disease-free survival following resection. To the best of our knowledge, this is the first study to examine the impact of stem cell marker expression on the prognosis of patients with colon cancer with peritoneal metastasis. Additionally, we found that patients with CD133-negative disease were more sensitive to chemotherapy than those with CD133-positive disease. Therefore, our data suggest that CD133 may be a useful clinical biomarker for the selection of patients who may receive optimal benefits from adjuvant chemotherapy following metastasectomy.

The impact of CD133 on survival has not yet been fully clarified. Based on the cancer stem cell theory, CD133-positive
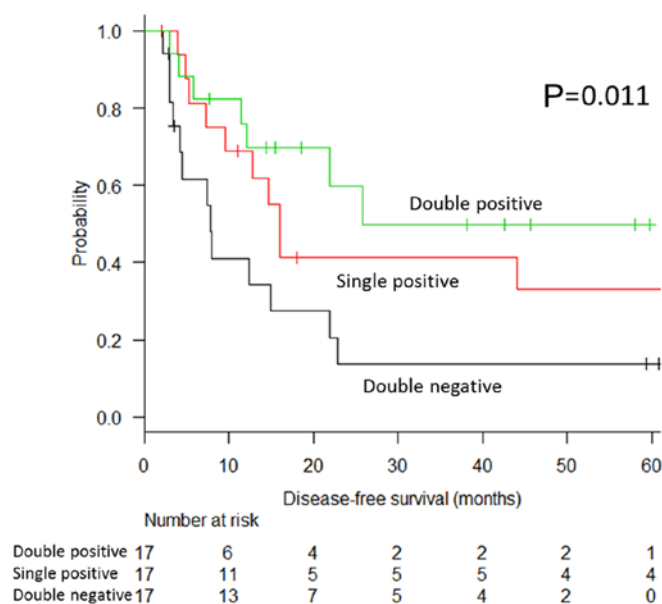

Figure 7. Association of the expression pattern of CD133 and disease-free survival. Double positive, both primary tumour and peritoneal nodules are CD133-positive; single positive, either primary tumour or peritoneal nodules are CD133-positive; double negative, both primary tumour and peritoneal nodules are CD133-negative.

tumours should have a worse prognosis. In fact, a number of studies, including 2 meta-analyses, have shown reduced overall survival in patients with CD133-positive colorectal cancer (14-17). However, studies on liver metastasis of colorectal origin have reported contradictory results, suggesting that the absence of CD133 expression is associated with a poor prognosis (7-9), which is consistent with our finding.

In the present study, we demonstrated that the CD133-negative group had a higher risk of recurrence following the resection of peritoneal metastasis. We employed 2 other stem cell markers, ALDH1 and Lgr5, in order to evaluate the impact of stemness, as CD133 cannot be designated as a definitive marker of stemness. For instance, while CD133-positive cells have been reported to have higher tumour initiation ability, CD133-negative subsets can also initiate tumours (18). Considering that a similar impact was not recognised in the expression of ALDH1 and Lgr5, the significance of CD133 identified in this study was presumed to be caused by CD133-specific factors other than stemness.

Although the findings on the function of CD133 are limited, *in vitro* studies have reported that E-cadherin expression is significantly lower (8) and that β1-integrin expression is higher (19) in CD133-negative cells than in CD133-positive cells. Other studies have found that CD133 is mainly expressed in well- and moderately-differentiated adenocarcinomas, and that CD133 negativity may reflect a more undifferentiated state (20,21), which is consistent with our findings. Therefore, CD133-negative cells may have a greater capacity to induce metastasis and recurrence than CD133-positive cells.

By contrast, we demonstrated in this study that the benefit of systemic chemotherapy was more evident in CD133-negative disease than in CD133-positive disease, which was consistent with the findings of previous studies (22,23). This difference in chemosensitivity may explain why the CD133-negative

group was not inferior to the CD133-positive group in terms of overall survival, despite the higher risk of recurrence.

To the best of our knowledge, this is the first study to examine the expression of cancer stem cell markers in nodules of peritoneal metastasis. We found that the expression rate of CD133 and ALDH1 was lower in peritoneal nodules than in primary tumours. The result was contrary to our assumption prior to the study, in that we had considered that the expression of cancer stem cell marker would be higher in peritoneal nodules, since each nodule should have a tumour initiation ability and peritoneal metastasis was known to be resistant to chemotherapy. The reason for the low expression is not clear; however, it may be related to the characteristics of CD133-negative cells that we described earlier. In particular, β1-integrin is known to be a key factor for developing peritoneal metastasis (24). ALDH1, in contrast, is a detoxifying enzyme contributing to differentiation and proliferation (25). It may represent the slow-growing nature of peritoneal nodules of colon cancer.

A major limitation of the present study was that none of our patients underwent peritonectomy or HIPEC, which is performed in specialised centres as an effective treatment for peritoneal metastasis of colorectal origin (26). Since the procedure has not been popular in the Japanese clinical setting, we have been performing macroscopic peritoneal nodule resection and have reported the survival benefit (27). Although our findings cannot be directly applied to patients who underwent peritonectomy and HIPEC, we speculate that the CD133 expression pattern may also be a useful indicator of recurrence risk in patients who undergo peritonectomy and HIPEC.

Another limitation is that the evaluation method of CD133 expression widely varies according to different reports, particularly in terms of the anti-CD133 antibody and the cut-off value (14,15). We believe that the immunohistochemical evaluation of CD133 is clinically practicable since the antibody is easily available and the analysis can be performed in many medical facilities. However, as variation in the evaluation method can cause inconsistent outcomes, standardised assessment criteria should be established. In addition, due to the small sample size of the study, the significance of ALDH1 and Lgr5 may be underestimated. Therefore, the findings of this study require confirmation in a larger patient cohort.

In conclusion, in this study, we demonstrated that a negative CD133 expression was a significant risk factor for post-operative recurrence following resection in patients with colon cancer with peritoneal metastasis. In addition, we also found that CD133-negative tumours were more sensitive to chemotherapy than CD133-positive ones. Our data suggest that CD133 expression may be a useful clinical biomarker in the treatment of peritoneal metastasis of colon cancer; however, the oncological function of CD133 requires further clarifications in future studies.

Acknowledgements

Competing interests

The authors declare that they have no competing interests.

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