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Original

Positive Relationship between CD133 Expression and Clinicopathologic Factors in Colorectal Cancer

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Abstract: The expression of the CD133 cancer stem cell marker correlates with metastasis and prognosis for many cancers, but no correlation has been established in colorectal cancer. We used immunohistochemical analysis to examine the relationship between CD133 expression and clinical malignancy factors such as lymph node metastasis and hepatic metastasis in colorectal cancer. The subjects of this study were 104 patients with colorectal cancer who were examined in our hospital and treated by surgical excision of the tumor between 2004 and 2007. Representative tissue sections were immunohistochemically stained using an anti-CD133 antibody. Patients showing staining of 50% or more of the tumor gland duct were classified into the CD133-positive group, which consisted of 36 patients. Those staining less than 50% of the tumor gland duct were classified into the CD133-negative group, which consisted of 68 patients. Patients with lymph node metastasis accounted for 63.9% of the positive group (23/36 patients) and 33.8% of the negative group (23/68 patients), and the difference was significant ($P=0.00331$). Patients with hepatic metastasis accounted for 27.8% of the positive group (10/36 patients) and 10.3% of the negative group (7/68 patients), and the difference was significant ($P=0.0218$). Classification of these patients according to cancer stage determined on the basis of the International Union Against Cancer (UICC) stage showed that five patients were in stage I, one patient in stage II, 20 patients in stage III, and 10 patients in stage IV in the positive group; and 20 patients were in stage I, 22 patients in stage II, 18 patients in stage III, and eight patients in stage IV in the negative group. There was a significant difference in the numbers of patients in each group ($P=0.000127$). Differences in the number of patients with lymphovascular invasion and those with venous invasion were also significant between the groups ($P=0.0248$ and $P=0.0292$, respectively). No significant differences were observed for any other factors. These findings indicate that the CD133-positive group has a higher risk of metastasis.

Key words: CD133, colorectal, immunohistochemistry, metastasis

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Introduction

A hierarchical model has been proposed recently in which a small population of cancer stem cells with self-renewal capability and multipotency have tumorigenic potential[1–3]. The existence of cancer stem cells was first demonstrated by the transplantation of human acute myeloid leukemia cells into severe combined immunodeficient (SCID) mice[4]. The existence of cancer stem cells has since been established by their separation from other cell types and identification and the analysis of their properties[5–9]. The current definition of cancer stem cells includes the following characteristics: 1) tumorigenicity, 2) the ability to form a cell group with low or no tumorigenicity, and 3) self-renewal capability[10].

CD133 is a five-transmembrane protein with a molecular mass of 117 kDa that is selectively localized in the cell membrane protrusions in hematopoietic and neural stem cells[11]. The expression of CD133 in cancer stem cells has been demonstrated using transplantation experiments in mice[8, 12]. CD133 is expressed in cancer stem cells in various solid cancers such as brain tumor[8, 12, 13], prostate cancer[14], kidney cancer[15], melanoma[16, 17], ovarian cancer[18], hepatocellular cancer[19–21], lung cancer[22], and colorectal cancer[23–25]. The relationship between CD133 expression and clinicopathologic findings in colorectal cancer has not been fully studied and no functional basis for its expression in these cells has been established.

In an investigation of colorectal cancer, we assessed the relationship between CD133 expression and clinicopathologic findings such as lymph node metastasis and hepatic metastasis.

Materials and Methods

Patients and specimens

We enrolled 144 patients with colorectal cancer in this study. These patients had undergone surgical excision at Showa University Hospital or Showa University Toyosu Hospital between 2004 and 2007. Patients with multiple cancers, or multiple primary cancers, or those given adjuvant therapy, were excluded from the study. Previously, Kojima *et al* used immunohistochemical analysis to show that there was no CD133 expression in 29 patients with poorly differentiated adenocarcinoma[26]. Therefore, patients with poorly differentiated adenocarcinoma were excluded from our study. Patients with lymph node metastasis and those with hepatic metastasis were diagnosed histologically. Excised tissue samples from patients meeting the selection criteria were fixed in 10% formalin and the tumor diameter and gross appearance were recorded. All of the tissue specimens were excised in accordance with the general rules of the Japanese Society for Cancer of the Colon and Rectum. After representative sections were embedded in paraffin, thin sections were prepared and stained with hematoxylin-eosin (HE) for histological evaluation of venous invasion by tumor cells.

Immunohistochemical staining

Immunohistochemical staining of representative colorectal cancer tissue sections for CD133 was performed using an anti-CD133 primary antibody (Miltenyi Biotec Inc., Auburn, CA, USA). Thin slices were heat-treated with ethylenediaminetetraacetic acid (EDTA) for 90 min after deparaffinization and hydrophilic treatment. Endogenous peroxidase activity was inhibited using 0.3% hydrogen peroxide solution. The primary anti-CD133 antibody was used at a dilution of 1 : 5 and reacted with the tissue antigen for 32 min. A secondary antibody was raised against biotinylated immunoglobulin and was labeled with avidin horseradish peroxidase (HRP). CD133 staining was visualized using a Ventana I-View DAB universal kit (Roche, Tokyo, Japan. The reaction was enhanced using copper sulfate. After nuclear staining with hematoxylin, the slices were mounted on slides.

Evaluation of CD133 immunopositivity

CD133 immunopositivity was evaluated using the assay method reported by Horst *et al*[27] Five medium power fields (×200) per section were viewed.

Patients showing staining of $<50\%$ of the tumor gland duct were classified into the negative group, and those showing staining of $\geq 50\%$ were classified into the positive group. Positive CD133 staining of the tumor gland duct was established by staining of the membrane surface of the ductal lumen or of debris in the tumor gland duct. Statistical analyses were carried out using the $M \times N$ χ^2 test and Welch's t-test with $P < 0.05$ indicating a significant difference.

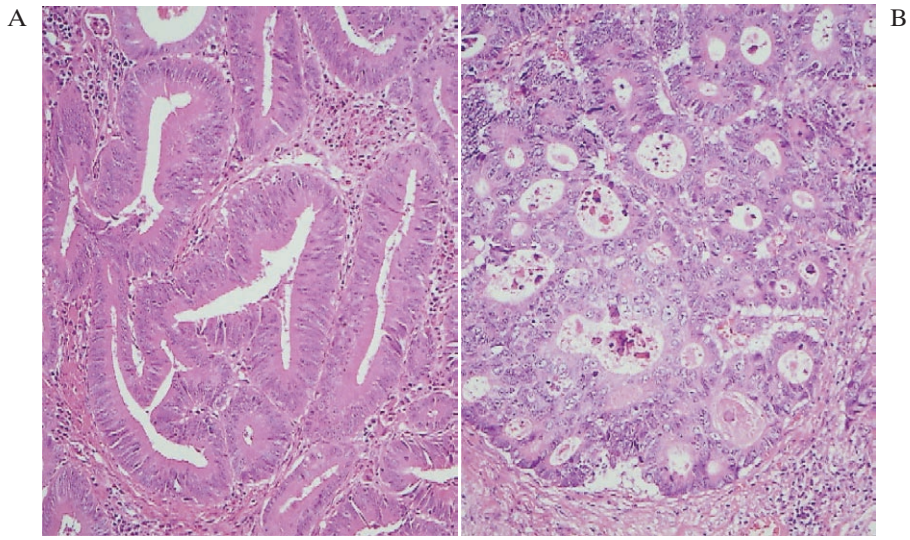
Results

Clinicopathologic findings

The clinicopathologic findings for the 104 selected patients are shown in Table 1. The patients consisted of 71 males and 33 females with a mean age of 69.2 ± 11.6 yr. The mean size of all the tumors was 45.4 ± 19.6 mm. The tumor onset sites were in the cecum in four patients, ascending colon in 18 patients, transverse colon in 12 patients, descending colon in 14 patients, sigmoid colon in 32 patients, and rectum in 24 patients. Thirty-nine patients had well-differentiated adenocarcinoma and 65 patients had moderately differentiated adenocarcinoma (Figs. 1A and 1B). Lymph node metastasis was observed in 58 patients (Fig. 2A) and hepatic metastasis was observed in 17 patients (Fig. 2B). There were 11 T1 patients, 20 T2 patients, 61 T3 patients, and 12 T4 patients as determined in accordance with tumor node metastasis (TNM) classification. Using the UICC classification, 25 patients were identified to be in stage I, 23 patients in stage II, 38 patients in stage III, and 18 patients in stage IV. Lymphovascular invasion was observed in 79 patients and venous invasion was observed in 76 patients.

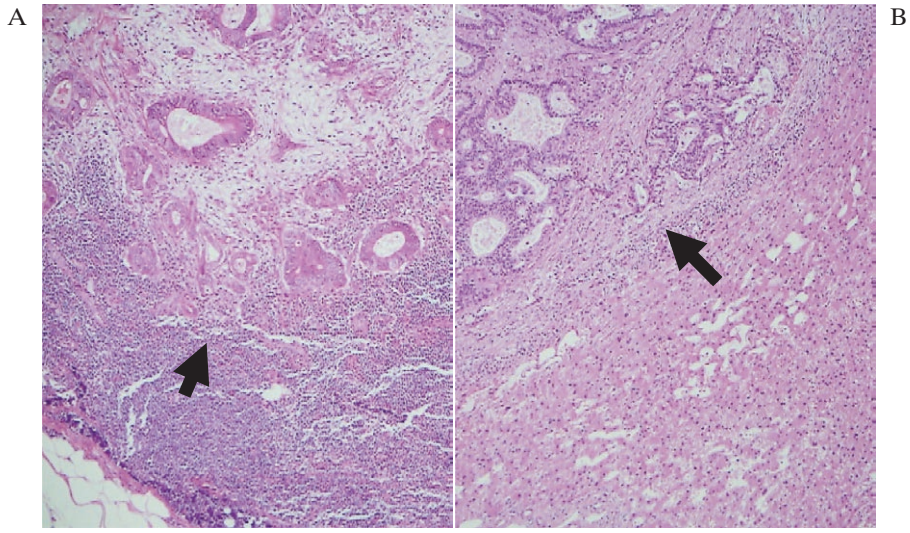
Table 1. Relationship between CD133 expression and clinicopathologic factors in colorectal cancer

	Total	Cd133 expression low	High	p value
n	104	68	36	
Age (years)				
Mean ± S.D	69.2 ± 11.6	67.5 ± 12.7	70.6 ± 9.75	0.211
Gender				
Male	71	43	28	
Female	33	25	8	0.131
Tumor size (mm)	45.4 ± 19.6	45.2 ± 19.0	45.7 ± 21.0	0.912
Location				
Cecum	4	2	2	
Ascending	18	14	4	
Trans	12	10	2	
D / C	14	8	6	
S / C	32	16	16	
Rectum	24	18	6	0.151
Histology				
well	39	28	11	
moderately	65	40	25	0.287
Depth of tumor				
pT1	11	10	1	
pT2	20	11	9	
pT3	61	41	20	
pT4	12	6	6	0.141
lymphnode metastasis				
negative	58	45	13	
positive	46	23	23	**0.00331**
Liver metastasis				
Negative	87	61	26	
Positive	17	7	10	**0.0218**
UICC Stage				
I	25	20	5	
II	23	22	1	
III	38	18	20	
IV	18	8	10	**0.000127**
lymphatic invasion				
Negative	25	21	4	
Positive	79	47	32	**0.0248**
Venous invasion				
Negative	28	23	5	
Positive	76	45	31	**0.0292**



A : Well-differentiated adenocarcinoma B : Moderately differentiated adenocarcinoma

Fig. 1. Tissue image (HE)

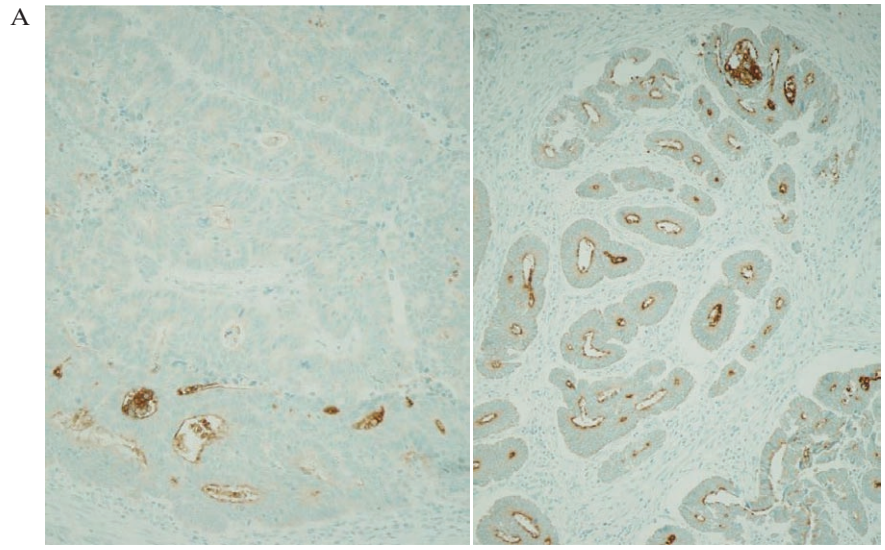


A : Patient with lymph node metastasis B : Patient with hepatic metastasis

Fig. 2. Tissue image (HE)

Assessment of correlation between CD133 expression and clinicopathological findings

There were 36 patients in the positive group and 68 patients in the negative group (Figs. 3A and 3B). Their mean ages were 70.6±9.75 yr for the positive group and 67.5±12.7 yr for the negative group, which is not a significant difference ($P=0.211$). The positive group consisted of 28 males and eight females, whereas the negative group consisted of 43 males



A : Patients with a CD133 positivity rate of ＜50% of the tumor gland duct were classified into the negative group.

B : Patients with a CD133 positivity rate of ≥50% of the tumor gland duct were classified into the positive group.

Fig. 3. Immunohistochemical evaluation of CD133 in colorectal cancer
The presence of CD133-positive cells in the tumor gland duct was determined on the basis of the staining of the membrane surface of the ductal lumen or staining of debris in the tumor gland duct.

and 25 females and there was no significant difference in the gender ratio between the two groups. The tumor diameters were 45.7±21.0 mm for the positive group and 45.2±19.0 mm for the negative group, which were not significantly different ($P=0.912$). The tumor onset sites were identified to be the cecum in two patients, ascending colon in four patients, transverse colon in two patients, descending colon in six patients, sigmoid colon in 16 patients, and rectum in six patients in the positive group. In the negative group, the tumor onset sites were in the cecum in two patients, ascending colon in 14 patients, transverse colon in 10 patients, descending colon in eight patients, sigmoid colon in 16 patients, and rectum in 18 patients. There was no significant difference in tumor onset site between the two groups ($P=0.151$). Analysis of the histological type of colorectal adenocarcinoma revealed that 11 patients had well-differentiated adenocarcinoma and 25 patients had moderately differentiated adenocarcinoma in the positive group. In the negative group, 28 patients had well-differentiated adenocarcinoma and 40 patients had moderately differentiated adenocarcinoma. There was no significant difference in histological type between the two groups ($P=0.287$). Patient distribution according to T classification identified one T1 patient, nine T2 patients, 20 T3 patients, and six T4 patients in the positive group. In the negative group there were 10 T1 patients, 11 T2 patients, 41 T3 patients, and six T4 patients. No significant difference in this distribution was observed between the two groups ($P=0.1408$). Lymph node metas-

tasis was observed in 63.9% (23 / 36 patients) of the positive group and in 33.8% (23 / 68 patients) of the negative group. The difference was significant between the two groups (P = 0.00331). Hepatic metastasis was observed in 27.8% (10 / 36 patients) of the positive group and 10.3% (7 / 68 patients) of the negative group, and the difference is significant (P = 0.0218). Patient distribution according to the UICC stage classification showed that five patients were in stage I, one patient in stage II, 20 patients in stage III, and 10 patients in stage IV in the positive group, whereas 20 patients were in stage I, 22 patients in stage II, 18 patients in stage III, and eight patients in stage IV in the negative group. A significant difference in patient distribution was observed between the two groups (P = 0.000127). Lymphovascular invasion was observed in 88.9% (32 / 36 patients) of the positive group and 69.1% (47 / 68 patients) of the negative group, and the difference was significant (P = 0.0248). Venous invasion was observed in 86.1% (31 / 36 patients) of the positive group and 66.2% (45 / 68 patients) of the negative group, and the difference was significant (P = 0.0292).

Discussion

A number of recent studies of colorectal cancer cells from CD133-positive patients have shown that the cells have different characteristics than those from CD133-negative patients. O'Brien *et al* isolated CD133-positive cells from surgically excised colorectal cancer specimens, transplanted them into SCID mice and found that they had more than 200-fold higher tumorigenicity than CD133-negative cells[23]. In addition, Ricci-Vitiani *et al* using subcutaneously transplanted clinical samples of colorectal cancer, found that CD133-positive cell populations formed tumors similar to the original tumors[24]. This also established that the passage of CD133-positive cells is possible[24]. The CD133-positive cell population in colorectal cancer cell lines has higher tumorigenicity with greater colony formation, invasion and growth in nude mice than CD133-negative cells[25].

Two previous studies of the clinicopathology of CD133-positive colorectal cancer tumors have also shown no significant relationship between CD133 expression and tumor diameter or tumor depth determined on the basis of T classification[26, 27]. Kojima *et al* demonstrated the uniformity of cancer stem cell distribution in tumors and observed no relationship between CD133 expression and tumor diameter or tumor depth[26]. These findings do not necessarily reflect the previously mentioned data using a colorectal cancer cell line.

In contrast to our findings, Kojima *et al* found no significant relationship between the presence of CD133-positive cells and lymph node metastasis, lymphovascular invasion or venous invasion[26]. Their interpretation may result from their analysis of the CD133 immunohistochemical data in which they used a cutoff for a positive signal of 10% for the entire tumor, whereas we set the cutoff at 50%. Therefore, their interpretation failed to identify a relationship between CD133-positive cells and vascular invasion and subsequent metastatic invasion. In the present study, we established that there is a significant relationship between the presence of CD133-positive cells and each of the clinicopathologic factors associated

with colorectal cancer. It has been established that CD133-positive cells are associated with lymphovascular invasion and lymph node metastasis in pancreatic cancer[28]. It has been proposed that the microenvironment of cancer stem cells is critical to their maintenance and self-renewal because of the close interactions between cancer stem cells and endothelial cells[29]. The results of the present study indicate that the microenvironment of cancer stem cells is favorable for lymph node and venous invasions, thereby leading to lymph node and hepatic metastases[28]. Although the specific prognosis of patients with colorectal cancer was not assessed, a review of the literature showed a significantly lower 5-year survival rate for CD133-positive patients, probably as a result of higher rates of lymphatic and hematogenous metastases[26, 27].

Here, we have reported on the significant relationship between the presence of CD133-positive cells and metastases of the lymph node and liver including vascular invasion. Not all CD133-positive cells have characteristics similar to cancer stem cells. O'Brien *et al* reported that only 1 / 262 of CD133-positive cells actually showed characteristics similar to those of cancer stem cells[23].

An additional cancer stem cell marker that is important in colorectal cancer is CD44. CD44 positivity group has characteristics of the cancer stem cell even if it is single ; further it has been reported that EpCAM-positive / CD44-positive / CD166-positive cell population more strongly show those characteristics[30].

Therefore, it is possible that CD133-negative / CD44-positive cell populations more efficiently identify cancer stem cells[31]. A systematic investigation using immunohistochemical analyses will be required to accurately identify markers strongly associated with the cancer stem cell population.

Conclusions

The presence of CD133-positive cancer stem cells is significantly related to the risk of vascular invasion and lymph node and hepatic metastases in colorectal cancer patients. CD133-positive cancer stem cells can be a malignancy indicator for colorectal cancer as in other cancers.

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