Expression of Cancer Stem Cell Markers CD133 and CD44 in Locoregional Recurrence of Rectal Cancer

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Abstract. Background: Recent findings suggest that cells with surface CD markers include cancer stem cells (CSCs) which can produce a cancer cluster, and that the presence of CSCs may be linked with prognosis. CD133 and CD44 are among the most useful markers for identification of colorectal CSCs. Materials and Methods: An immunohistological analysis of CD133 and CD44 was performed using tissue from cases shown to be locoregionally recurrent or non-recurrent clinico-pathologically. Results: The CD133-positive rates were 38.7% and 59.23% in non-recurrent and recurrent cases, respectively, and the CD44-positive rates were 35.5% and 44.4%, respectively. Expression of the CD markers had no correlation with other clinicopathological factors. The prognosis of patients who were positive for both markers was significantly worse than that of other patients. Conclusion: These results suggest that detection of CD133 and CD44 can provide useful information for selection of treatment and performance of intensive follow-up of colorectal cancer.

Colorectal cancer is the second most common cancer and a major cause of mortality (1). Locoregional recurrence in the pelvis occurs frequently after rectal cancer surgery, and early diagnosis and initiation of therapy are desirable since recurrent cases have a poor prognosis. Thus, the 5-year survival rate for colorectal cancer without lymph node involvement is 66-80%, while the presence of distant metastases reduces the survival rate by up to 7% (2). The cancer is resectable in 70-80% of newly diagnosed patients with localized disease, but most die within 5 years from advanced recurrence or metastasis after surgery or adjuvant chemotherapy.

The cancer stem cell (CSC) theory is a particularly interesting concept in current cancer research. According to this theory, cancer clusters cannot be considered as simple monoclonal expansions of cancer cells with equivalent functions. In contrast, regardless of their monoclonal origin, only a small minority of cancer cells are proposed to have the capacity to maintain malignancy (3, 4). The CSC theory originated from hematology and is now beginning to be considered in terms of CD markers of CSCs. Several CD molecules have been identified as CSC markers. CD133 (PROML1 or prominin) is a cell surface antigen that has recently been recognized as a potential CSC marker in brain, colon and prostate cancer (5-8). CD44 (homing cell adhesion molecule) is a cell surface glycoprotein that is expressed on lymphocytes, monocytes and granulocytes, and has been recognized as a CSC marker in breast, pancreas, and head and neck cancer (9-11).

CD133 and CD44 appear to be useful markers for isolation and further characterization of colorectal CSCs. However, the relationships of recurrent cancer with and without these markers with other clinicopathological features are unknown. Moreover, there have been few studies of the prognosis of cases of recurrent rectal cancer based on the presence or absence of CD markers.

In this study, the relationship of locoregional recurrence after rectal surgery with immunohistological detection of CD133 or CD44 was examined in rectal cancer samples. CD133+ and CD44+ cancer cells were identified in the tumor samples, and these findings were compared with the clinicopathologic features of recurrent and non-recurrent cases.

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Materials and Methods

Clinical samples. Samples were obtained from patients with histologically proven colorectal cancer who underwent surgical resection between 1995 and 2006 at the Kyoto Prefectural University of Medicine. Several of these patients died in the immediate postoperative period and were excluded from the study. Follow-up by outpatient consultations or by telephone and mail was performed until death or the cutoff date. Several cases were lost to follow-up and were also excluded. The final analysis included rectal cancer specimens from 58 cases, including 27 in which surgery was performed for resection of local recurrent lesions in the pelvis, and 31 without recurrence after the initial rectal surgery. The study was approved by our Institutional Ethics Committee.

Immunohistochemistry. Immunohistochemical staining was carried out using 5-μm sections of formalin-fixed, paraffin-embedded tumor samples. Detection of CD133 and CD44 in these samples was performed with AC133 (CD133) antibody, purified rabbit polyclonal antibody (Pab) (1:50, clone RB1784; ABGENT, San Diego, CA, USA) and anti-CD44 mouse mAb (1:50, clone BU75; Ancell, Bayport, MN, USA) as the respective primary antibodies. For antibody staining, endogenous peroxidase was blocked using 0.3% hydrogen peroxide for 30 min and primary antibody incubation was carried out at room temperature for 120 min. Vectastain ABC-Kits Elite Universal (Vector Laboratories, Burlingame, CA, USA) were used for detection and slides were developed with Liquid DAB Substrate (Dako, Japan).

Evaluation of immunostained tissue sections of colorectal adenocarcinomas was performed for the edge of the tumor. Positive tumor glands were defined based on cytosolic staining (Figure 1A) and membranous staining (Figure 1B) of CD44 and CD133, respectively.

Statistical analysis. Cross-tabulations were calculated by Chi-square test (or Fisher exact test) and t-test. Kaplan-Meier analysis was used to evaluate overall survival, and CD-negative and -positive cases were compared by log-rank test. All statistical analyses and graphics were performed with JMP 7.0.1 package (SAS Institute Inc., Cary, NC, USA). Statistical significance was accepted at the p < 0.05 level.

Results

CD133 and CD44 were both detected immunohistochemically in colorectal cancer specimens. CD marker expression in tissue samples from rectal cancer and from recurrent lesions was found in the cytosol and in the membranous region at the apical luminal surface, as described by Horst et al. (12). Therefore, the in situ expression patterns of our samples were consistent with previously published data on CD-positive cell phenotypes.

The CD133+ rate did not differ significantly between the non-recurrent and recurrent groups (38.71% vs. 59.20%, p=0.117). Similarly, the CD44+ rate did not differ significantly between the respective groups (35.48% vs. 44.40%, p=0.487). However, the percentage of cases that were both CD133+ and CD44+ tended to be higher in the recurrent group (19.35% vs. 40.74%, p=0.073) (Figure 2).

Therefore, a combination of CD markers may be more effective for differentiating between recurrent and non-recurrent cases, and for predicting a risk of locoregional recurrence after rectal cancer surgery. Comparison of the CD marker status with clinicopathologic features showed no correlation with tumor size, age, gender, depth of the tumor, lymphatic and blood vessel invasion, and LN metastasis (Table I).

Next, we examined the correlation of expression of CD markers with patient prognoses using Kaplan-Meier analysis. The median and mean overall survival periods in the recurrent group were 23.6 and 26.9 months, respectively (range: 2.6-71 months). Five-year overall survival was shorter in the CD44+ group than in the CD44- group (p=0.088), and was significantly shorter in patients who were both CD133+ CD44+ compared to all other patients (p=0.0225) (Figure 3). These results show that a positive status for both CD markers is associated with a significant difference in prognosis after rectal cancer surgery. In contrast, neither CD marker alone was predictive of prognosis. Thus, combined analysis of CD133 and CD44 may be more effective for establishing the risk of a poor prognosis after rectal cancer surgery.

Discussion

The field of CSCs is relatively new and many questions remain unanswered. Studies on solid tumors have begun to provide experimental evidence that supports the presence of CSCs. It has been proposed that a rare subpopulation of cells within the cancer cluster has the potential to self-renew, differentiate and maintain cancer growth (13-15); and recent studies have shown that leukemia (16, 17) and several solid tumors, such as gastric (18), liver (19, 20), pancreatic (10), prostate (21-23), breast (9, 24), lung (25), glioblastoma (7), and colorectal (5, 6, 26) tumors, contain such subsets of CSCs. The current consensus definition describes a CSC as a cell within a tumor that can renew itself and can develop heterogeneous cancer cells that form the cancer body (27-29). This definition is consistent with the use of ‘cancer-initiating cells’ as an alternative term to describe putative CSCs (5, 6, 9, 30).

CD133 and CD44 have been described as stem cell markers for colorectal cancer in several reports, and use of CD133 and CD44 as markers to identify CSCs has been suggested for mammary (9, 24), brain (7), liver (19, 20) and prostate (22, 23) cancer. CD133 (AC133), a glycoprotein containing five transmembrane domains, is expressed on CD34+ hematopoietic stem cells derived from human fetal liver and bone marrow (31). CD133+ cells from glioblastoma (7) and pancreatic cancer (32) have the capacity to form tumors in mice more efficiently compared to residual cells from the total tumor population. CD44 has been used as a marker of CSCs in breast (9, 24), head and neck (33) and...
prostate cancer (22, 23). Physiologically, CD44 is a key cell membrane glycoprotein that has a role in cell-matrix adhesion and lymphocyte activation (34).

In this study, we demonstrated that CD133+ or CD44+ cells are clearly present in some colorectal tumors, but the actual CSCs among these cells cannot be determined using these or other markers. Thus, although CD133 and CD44 are currently two of the best markers for CSCs, it is clear that not every CD133+ or CD44+ cell is a CSC. For example, it has been calculated that only 1 in 262 CD133+ colon cancer cells actually has the capacity for cancer initiation (5).

Figure 1. Immunostaining for CD markers was performed using whole tissue sections of colorectal adenocarcinomas. Positive tumor glands were defined by cytosolic staining or luminal surface staining of glands. A: Loupe and microscope image (x400) of cytosolic staining by CD44 antibody. B: Loupe and microscope image (x400) of surface staining by CD133 antibody.

Figure 2. The CD-133 positive rate of the non-recurrent and recurrent groups (38.7% (12/31) vs. 59.2% (16/27), p=0.117) did not differ significantly, nor did the CD44-positive rate of the respective groups (35.5% (11/31) vs. 44.4% (12/27), p=0.487). Both CD133- and CD44-positivity tend to be higher in the recurrent group (19.4% (6/31) vs. 40.7% (11/27), p=0.073). P-values were calculated by cross-tabulations.
We investigated the expression of CD markers in rectal cancer specimens using an immunohistochemical approach. The localization of CD marker-positive colorectal cancer cells has yet to be verified in situ, and organ-specific expression patterns of these markers require further clarification. Horst et al. found that the CD133 antigen is located at the luminal surface of epithelial tumor glands with shedding into the lumina (12), whereas more recent staining results suggest cytoplasmic expression of CD133 in pancreatic cancer samples (32, 35).

In the present study, there was a trend for recurrent cases being positive for both CD133 and CD44 compared to non-recurrent cases. This result suggests that cancer cells with these markers might play a role in recurrence, and CD44 has been proven to be a strong marker for stem cells in colon cancer (26). The role of CD44 as a CSC marker was further investigated in gene expression profiling by Polyak et al., with the finding that CD44+ breast cancer cells had a gene profile similar to that of stem cells (36). Elevation of mRNA levels for CD133 is also predictive of a tendency for recurrence in stage IV colon cancer (37). Increased CD133 expression has also been correlated with a higher tumor grade, advanced disease stage, and elevated serum alpha-fetoprotein levels.

In our study, overall survival of CD44-positive patients tended to be shorter than that of other patients, although without any significant difference. The lack of statistical significance may be due to the small number of recurrent cases after rectal surgery, and the importance of CD44 may become clearer with accumulation of more cases. Only a few studies have examined the prognostic impact of the presence of CD133+ cells in solid tumors and the results are contradictory (38-40). For example, in patients with glioma, the proportion of CD133+ cells in cancer clusters was a significant prognostic factor and an indicator of recurrence and malignant progression (40). In pancreatic cancer patients, Maeda et al. showed that CD133 expression correlated with lymph node metastasis and survival (35). However, Immervoll et al. did not find a significant link between CD133 expression and the prognosis of patients with pancreatic cancer (42).

A striking aspect of the current study was that the overall survival of patients who were positive for both CD133 and CD44 was significantly shorter than that of all other patients. Our results also suggest that both CD markers in combination may be an indicator of poor prognosis after rectal cancer surgery. Therefore, CD133+ or CD44+ cells may have a tendency to aggravate prognosis, and combination analysis of these markers may provide useful information for selection of treatment and performance of intensive follow-up.

### Table I. Univariate analysis of the relationships of CD markers with tumor size, age, invasive depth of the tumor, lymphatic and vascular invasion, and lymph node (LN) metastasis.

<table>
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<tr>
<th></th>
<th>CD44 (%)</th>
<th>p-Value</th>
<th>CD133 (%)</th>
<th>p-Value</th>
<th>Both (%)</th>
<th>p-Value</th>
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<tr>
<td>Tumor size (mm)*</td>
<td>107.0±30.2</td>
<td>0.429</td>
<td>132.7±25.7</td>
<td>0.595</td>
<td>107.0±30.2</td>
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<td>Age (years)*</td>
<td>65.0±3.3</td>
<td>0.173</td>
<td>63.8±2.9</td>
<td>0.231</td>
<td>64.9±3.5</td>
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<tr>
<td>Male</td>
<td>8 (66.7)</td>
<td>0.139</td>
<td>11 (68.8)</td>
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<td>7 (63.6)</td>
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<td>5 (31.2)</td>
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<td>4 (36.4)</td>
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<td>Differentiated</td>
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<td>0.628</td>
<td>14 (87.5)</td>
<td>0.371</td>
<td>9 (81.8)</td>
<td>1.000</td>
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<td>2 (12.5)</td>
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<td>2 (18.2)</td>
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<td>se</td>
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<td>2 (12.5)</td>
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<tr>
<td>si</td>
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<td></td>
<td>5 (31.2)</td>
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<td>3 (27.3)</td>
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<tr>
<td>Lymphatic invasion</td>
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<tr>
<td>Present</td>
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<td>14 (87.5)</td>
<td>0.371</td>
<td>10 (90.9)</td>
<td>0.619</td>
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<td>2 (12.5)</td>
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<td>1 (9.1)</td>
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<td>Vascular invasion</td>
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<tr>
<td>Present</td>
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<td>12 (75.0)</td>
<td>1.000</td>
<td>9 (81.8)</td>
<td>0.662</td>
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<tr>
<td>Absent</td>
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<td></td>
<td>4 (25.0)</td>
<td></td>
<td>2 (18.2)</td>
<td></td>
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<tr>
<td>LN metastasis</td>
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<tr>
<td>Present</td>
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<td>0.441</td>
<td>8 (50.0)</td>
<td>0.696</td>
<td>4 (36.4)</td>
<td>0.696</td>
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<tr>
<td>Absent</td>
<td>8 (66.7)</td>
<td></td>
<td>8 (50.0)</td>
<td></td>
<td>7 (63.6)</td>
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*Mean±standard deviation (SD). Two-tailed t-test. mp: Muscularis propria, ss: sub serosa, se: serosa, si; invasion directly to other organ.
Figure 3. Survival curves for patients who were positive for cancer stem cell markers. A: The prognosis of CD133-positive cases did not differ significantly from that of CD133-negative cases (5-year survival rate: 13.3% vs. 20.0%; p=0.5748). B: CD44-positive cases had poorer survival than CD44-negative cases (5-year survival rate: 0.0% vs. 28.6%; p=0.0882). C: Patients who were positive for CD44 and CD133 showed a significantly poorer prognosis than other patients (5-year survival rate: 0.0% vs. 25.0%; p=0.0225).

References


