Cancer stem cell-related factors are associated with the efficacy of pre-operative chemoradiotherapy for locally advanced rectal cancer

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Abstract. Pre-operative chemoradiotherapy (CRT) is an important neoadjuvant therapy for locally advanced rectal cancer. In the present study, we investigated the factors that influence the efficacy of pre-operative CRT in locally advanced rectal cancer. We divided 50 patients with locally advanced rectal carcinoma treated with pre-operative CRT into two groups according to the grade of tumor response to pre-operative CRT: low-sensitivity group and high-sensitivity group. As candidates for the prediction of sensitivity to pre-operative CRT, clinicopathological factors and 12 biomarkers, including factors related to tumor growth, cell cycle, apoptosis, tumor stroma and cancer stem cells, were examined immunohistochemically in 48 resected specimens. Thirty-one tumors showed high sensitivity and 19 showed low sensitivity to pre-operative CRT. The status of stem cell-related factors, CD133 and CD24, was significantly associated respectively with sensitivity to pre-operative CRT (P=0.003, P=0.029). In 10 tumors positive for both CD133 and CD24, low sensitivity to CRT was found in 9 (90%), whereas in 16 tumors negative for both CD133 and CD24, low sensitivity was found in 3 (19%). Other pathological parameters were not associated with tumor response to pre-operative CRT. In conclusion, overexpression of cancer stem cell-related factors, CD133 and CD24, is associated with the sensitivity of locally advanced rectal cancer to pre-operative CRT.

Introduction

Colorectal cancer is a leading cause of morbidity and mortality in developed countries (1). In Japan, an increasingly Westernized diet has led to a high incidence of colorectal cancer. Patients with rectal cancers are known to have an increased rate of local recurrence and decreased survival time compared to patients with tumors of the colon, a result due primarily to the surgical constraints imposed by the location of the rectum within the pelvis (2).

Pre-operative chemoradiotherapy (CRT) is a neoadjuvant therapy for locally advanced rectal cancer that reduces the incidence of local recurrence and improves survival (3). Therefore, CRT is widely used in many countries of the world. However, several tumors show a marked response to CRT, whereas others do not. Furthermore, several adverse events related to CRT, such as enteritis, anorexia, cardiac/thromboembolic events, radiation dermatitis and hematologic toxicity, were reported to occur at frequencies of 6-43% (4). Thus, pre-operative indicators of chemoradiosensitivity are required to avoid unnecessary application of pre-operative CRT, yet little is known about potential biological markers that may be associated with response to pre-operative CRT.

Recently, the discovery of rare subpopulations of cancer stem cells has created a new focus in cancer research. The heterogeneity of tumors can be explained by the concept of cancer stem cells supported by anti-apoptotic signaling. There are a few reports on cancer stem cells related to chemoradiation resistance (5,6). Therefore, in this study we investigated the factors, including cancer stem cell-related factors, that influence the sensitivity of locally advanced rectal cancer to pre-operative CRT using surgical resected specimens to consider tumor heterogeneity.

Materials and methods

Patients. A total of 50 patients with locally advanced rectal carcinoma were treated with pre-operative CRT and surgical resection at the Department of Surgery I, Oita University Faculty of Medicine, or associated institutions (Beppu Medical Center, Nakatsu Municipal Hospital, Oita Prefectural Hospital and Nankai Hospital) between January 2000 and May 2010. Tumors were located at the middle or lower third of the rectum and were diagnosed as clinical stage T2, T3 or T4, Nx and M0 (UICC TNM Classification of Malignant Tumours, 2009). T stage was determined by computed tomography (CT) scan or endoscopic ultrasonography. No

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distant metastases were detected on plain chest X-rays or CT scans. Thirty-nine patients were treated with pre-operative CRT and another 11 patients were treated with pre-operative radiotherapy (RT) alone. The total dose of radiation in most cases was 45 Gy within 6 weeks, usually 1.5 Gy per treatment, five times per week. The total dose range was 40-50 Gy. Several chemotherapy regimens were used in the patients treated with CRT: TS-1 (80 mg/m^2) in 21 patients, 5-fluorouracil (5-FU)-based in 5 patients, tegafur/uracil (UFT) and leucovorin or UFT alone in 8 patients, and tegafur in 5 patients. Curative surgery that included total mesorectal excision was performed in all patients after an interval of approximately 4 weeks following completion of pre-operative treatment. Patient informed consent and approval of the local ethics committee was obtained prior to the study.

Immunohistochemistry. A total of 12 biomarkers were chosen as candidate predictive factors for the efficacy of pre-operative CRT (7-13). These factors included tumor growth-related factors, epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor-2 (HER2); cell cycle-related factors, p53, p21, Ki-67 and Bcl-1; apoptosis-related factors, Bcl-2 and apoptosis protease-activating factor-1 (APAF-1); tumor stroma-related factors, vascular endothelial growth factor (VEGF) and macrophage migration inhibitory factor (MIF); and cancer stem cell (tumor initiating cell)-related factors, CD133 and CD24. Postoperative resected specimens were used for immunohistochemistry.

Paraffin-embedded sections of tumor tissue from the resected rectum were cut at a thickness of 4 μm, deparaffinized in xylene and rehydrated. Endogenous peroxidase activity was blocked with 3% hydrogen peroxidase for 10 min. For antigen retrieval, sections were autoclaved at 121˚C in 10 mM citrate buffer, pH 6.0, for 10 min. Sections were then treated with primary antibodies. Immunostaining was performed by the avidin-biotin-peroxidase complex technique using a Histofine SAB-PO (Multi) kit (Nichirei Co., Tokyo, Japan) and diaminobenzidine for the visualization of the binding antibodies (14). The following primary antibodies were used: EGFR (clone EGFR113, 1:100; Lab Vision Inc., Fremont, CA, USA) (15); p53 (clone DO-7, 1:50; DakoCytomation, Glostrup, Denmark); p21 (clone SX118, 1:40; DakoCytomation); Ki-67 (clone MIB-1, 1:50; DakoCytomation); Bcl-1 (clone SP4; Nichirei Co.) (16); Bcl-2 (clone 124, 1:40; DakoCytomation); APAF-1 (NCL-APAF-1, 1:20; Novocastra, Newcastle, UK) (17); VEGF (VEGF A-20, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) (18); MIF (FL-115, 1:200; Santa Cruz Biotechnology) (13); CD133 (ab19898, 1:200; Abcam, Tokyo, Japan) (19); and CD24 (clone SN3b, 1:100; Lab Vision Inc.) (20). Immunohistochemistry for HER2 was performed with HercepTest (DakoCytomation) (21). Negative controls were treated identically, omitting the primary antibodies. Tumor positivity for a given marker was evaluated using a pre-determined cut-off of 10% (the average of the percentage of tumor cells stained in five fields at x100 magnification: ≤10% tumor cell staining, negative; >10%, positive) according to previous studies (7,8,22). For Ki-67 immunoreactivity, staining was considered positive at >60% (23). Staining was assessed in the nucleus for p53, p21, Ki-67 and Bcl-1, and in the cytoplasm for EGFR, APAF-1, VEGF, MIF, CD133 and CD24. Immunoreactivity for Bcl-2 and HER2 expression was assessed in both the cytoplasm and/or the cell membrane. Staining intensity was not evaluated.

Table I. Patient and treatment characteristics.

Characteristic	No. of patients (n=50)	%
Age (years)		
Median	64	
Range	40-83	
Gender		
Male	37	74
Female	13	26
Surgery		
Total pelvic exenteration	1	14
Abdominoperineal resection	24	48
Sphincter-preserving operation	19	38
Macropathology		
Circumscribed	41	82
Infiltrative	9	18
Histology[a]		
Well differentiated	9	19
Moderately differentiated	31	66
Poorly differentiated	3	6
Mucinous	4	9
T-category[a]		
pT1	2	4
pT2	8	17
pT3	27	57
pT4	10	21
N-category		
pN0	38	76
pN$^+$	12	24
Vessel invasion		
Negative	25	50
Positive	25	50
Tumor response (CRT sensitivity)		
High sensitivity	31	62
Low sensitivity	19	38

[a]Three tumors were excluded from the pathological study due to complete pathologic tumor regression. CRT, chemoradiotherapy.

Classification of response to pre-operative CRT. Tumor response to pre-operative CRT was evaluated pathologically on postoperative specimens according to the evaluation of the standard of therapeutic effect provided in the General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus edited by the Japanese Society for Cancer of the Colon and Rectum (24). According to these standards, evaluation of the therapeutic effect was categorized according to five grades: grade 0, absence of regressive changes; grade 1a, regressive change of tumor <1/3; grade 1b, regressive change of

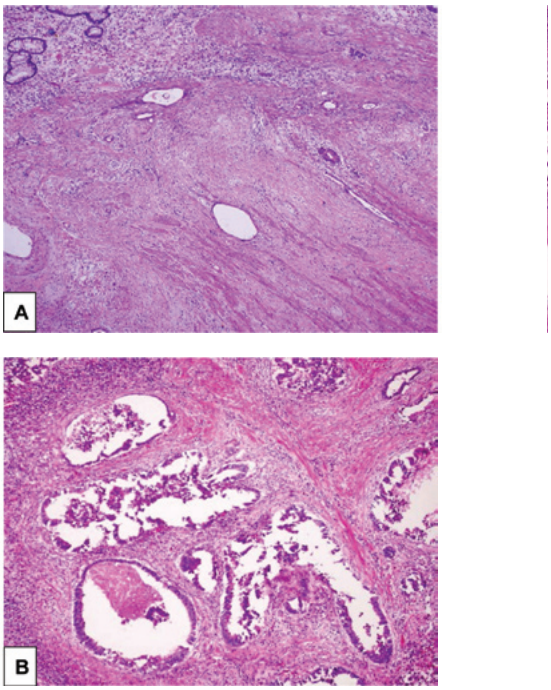

Figure 1. Photomicrographs indicating classification of the pathological response of pre-operative CRT in rectal cancer. (A) High-sensitivity case in which most tumor cells are replaced by fibrosis accompanying the infiltration of inflammatory cells (H&E stain; original magnification, x40). (B) Low-sensitivity case in which most tumor cells remain with mild tumor necrosis and regressive change (H&E stain; original magnification, x40).

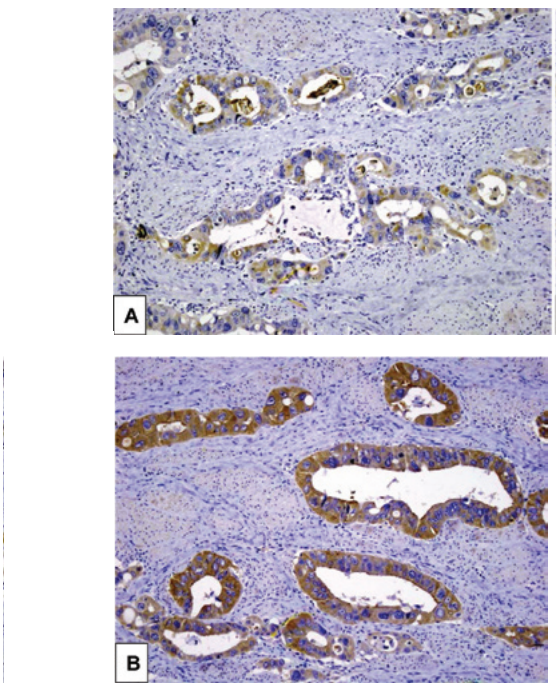

Figure 2. Photomicrographs showing immunohistochemical staining of CD133 and CD24 in rectal carcinoma. (A) Cytoplasmic expression of CD133 in tumor cells is observed (original magnification, x200). Glioblastoma tissue sections were used as a positive control. (B) Strong cytoplasmic expression of CD24 in tumor cells is observed (original magnification, x200). Ovarian serous adenocarcinoma tissue sections were used as a positive control.

tumor <2/3; grade 2, regressive change of tumor >2/3; grade 3, absence of residual tumor cells. We considered grades 0 or 1a to indicate low sensitivity and grades 1b, 2 or 3 to indicate high sensitivity to pre-operative CRT (Fig. 1).

Statistical analysis. For statistical comparisons of patient characteristics between the two groups (low sensitivity and high sensitivity), the Chi-square test, the Fisher's exact probability test or the unpaired t-test was used. A value of $P<0.05$ was considered statistically significant. All analyses were performed with SPSS Software (version 11.0) (SPSS Japan Inc., Tokyo, Japan).

Results

Patient and tumor characteristics. There were 37 (74%) men and 13 (26%) women included in the study. The median age was 64 years (range 40-83). Abdominoperineal resection was performed in 24 (48%) patients and a sphincter-preserving operation was performed in 19 (38%) patients. Macroscopic findings showed 82% of the tumors to be circumscribed tumors and, histologically, most (85%) of the tumors were of the well or moderately differentiated type. Lymph node metastasis was observed in 12 (24%) patients. Vessel invasion was observed in 25 (50%) patients. On the basis of the classification of responses to pre-operative CRT, 31 tumors showed high sensitivity and 19 tumors showed low sensitivity to pre-operative CRT (Table I).

Status of response to CRT according to various clinical parameters. Gender, age, macropathology, location, histology, N-category and surgery were not associated with tumor response (Table II). Of the 10 patients with pT1-2 tumors, 9 showed high sensitivity. The number of pT3-4 tumors showing high sensitivity was nearly equal to those showing low sensitivity ($P=0.034$). Of the tumors negative for vessel invasion, 21 of 25 showed high sensitivity, whereas 15 of 25 tumors positive for vessel invasion showed low sensitivity ($P=0.003$).

Response rates according to various pathological parameters. Factors related to tumor growth, the cell cycle, apoptosis and tumor stroma were not associated with tumor response (Table III). Only factors related to cancer stem cells (tumor-initiating cells) were associated with tumor response. A significant association was found between the resistance of the tumor to treatment and negative CD133 status ($P=0.003$), and there was a significant statistical correlation between the resistance of the tumor to treatment and positive CD24 status ($P=0.029$). In the high-sensitivity tumors, 3 tumors that had complete pathologic tumor regression were excluded from the pathological study (histology and T-category in Tables I and II) and immunohistochemical analysis since the resected specimens did not contain cancer cells (Fig. 2).

Response rates based on combinations of CD133 and CD24. When both CD133 and CD24 were positive, 9 of 10 (90%) tumors showed low sensitivity, whereas when both CD133 and CD24 were negative, 3 of 16 (19%) tumors showed low sensitivity (Table IV). Co-overexpression of CD133 and CD24 was associated with low sensitivity ($CD133^+$ and $CD24^+$ vs. others, $P=0.001$). Negative expression of both CD133 and

Table II. Response according to various clinical parameters.

Parameter	High sensitivity (n=31)	Low sensitivity (n=19)	P-value
Gender			0.481
Male	24	13	
Female	7	6	
Age (years)			0.635
Median	64	65	
Range	44-82	40-83	
Macropathology			0.715
Circumscribed	26	15	
Infiltrative	5	4	
Location			0.273
Upper	4	5	
Lower	27	14	
Histology[a]			0.102
Well/moderate differentiation	26	14	
Poor/mucinous differentiation	2	5	
T-category[a]			0.034
pT1/2	9	1	
pT3/4	19	18	
N-category			0.764
pN0	24	14	
pN1,2	7	5	
Vessel invasion			0.003
Negative	21	4	
Positive	10	15	
Surgery			0.464
LAR/Lap. LAR	13	6	
APR/Lap. APR	18	13	

[a]Three tumors were excluded from pathologic study due to complete pathologic tumor regression. APR, abdominoperineal resection (including total pelvic exenteration); Lap., laparoscopic; LAR, low anterior resection (including sphincter-preserving operation).

CD24 was associated with high sensitivity ($CD133^-$ and $CD24^-$ vs. others, P=0.030).

Discussion

The present study demonstrated that co-overexpression of cancer stem cell-related factors, CD133 and CD24, was significantly associated with locally advanced rectal cancer exhibiting low sensitivity to pre-operative CRT. This result suggests that these two biomarkers may influence sensitivity to pre-operative CRT.

In this study, we used resected specimens from patients who had been treated with pre-operative CRT. For identifying factors which predict the efficacy of CRT before treatment, the use of pre-treatment biopsy specimens is advisable. However, there is heterogeneity in the tumor (5). Therefore, biopsy specimens were not used, and resected specimens were used to investigate the entire tumor specimen.

For the evaluation of CD133 and CD24 expression, immunostaining was classified using the 10% cut-off scoring system. Although one report set the cut-off value to 50%, we adopted the standard system as it has been widely used in many studies. Expression of CD133 and CD24 was distributed evenly within the resected tumors. In the localization of staining, membranous expression of CD24 without cytoplasmic positivity was detected, but we did not include it as being indicative of positive expression.

The concept of cancer stem cells which has been proposed in the field of blood cancer (25) has been adjusted to address solid tumors, such as those of colorectal cancer (26). The fundamental cancer stem cell concept assumes that cancer cells exhibit a hierarchy, as do normal cells, and that a small fraction of cancer cells are maintained as 'cancer stem cells', which have the ability of self-renewal and differentiation (27). Cancer stem cells have recently been proposed to be the cancer-initiating cells that are responsible for tumorigenesis and for contributing to drug resistance in cancer (28). Although a comparatively large number of studies have been reported concerning cancer stem cells and resistance to either chemotherapy or radiotherapy in various cancers, there are few studies available concerning cancer stem cells and resistance to CRT (5).

Table III. Response according to various pathological parameters.

Biomarker	High sensitivity (n=28)	Low sensitivity (n=19)	P-value
HER2			1.000
+	1	0	
-	27	19	
EGFR			0.453
+	4	5	
-	24	14	
VEGF			0.119
+	21	18	
-	7	1	
MIF			0.770
+	13	8	
-	15	11	
p53			0.137
+	24	19	
-	4	0	
p21			0.143
+	5	7	
-	23	12	
Ki-67			0.739
+	19	12	
-	9	7	
Bcl-1			1.000
+	7	4	
-	21	15	
Bcl-2			0.435
+	16	13	
-	12	6	
APAF-1			0.119
+	21	18	
-	7	1	
CD133			0.003
+	2	9	
-	26	10	
CD24			0.029
+	14	16	
-	14	3	

+, positive expression; -, negative expression.

Table IV. Response according to combinations of CD133 and CD24.

Case	High sensitivity (n=28)		Low sensitivity (n=19)	
	No.	%	No.	%
$CD133^+$ and $CD24^{+a}$	1	10	9	90
$CD133^+$ and $CD24^-$	1	100	0	0
$CD133^-$ and $CD24^+$	13	65	7	35
$CD133^-$ and $CD24^{-b}$	13	81	3	19

[a]($CD133^+$ and $CD24^+$) vs. others, P=0.001. [b]($CD133^-$ and $CD24^-$) vs. others, P=0.030.

CD133 and CD24 have been reported as cancer stem cell markers of colorectal cancer in previous studies (26,29,30). CD133 is a 5-transmembrane glycoprotein of 865 amino acids with a total molecular weight of 120 kDa. CD133 antigen expression has been found in such various undifferentiated cells as hematopoietic stem cells (31) and fetal brain stem cells (32). In cancer cells, CD133 has been found to be expressed on cancer stem or tumor-initiating cells in cancers, such as leukemia (33), brain tumors (34) and colorectal cancer. CD24 consists of a small protein core comprising 27 amino acids, which is extensively glycosylated and is bound to the cell membrane via a phosphatidylinositol anchor (35). Several reports have shown that CD24 is expressed in several solid tumors, such as those of small-cell lung cancer and neuroblastoma (36,37), but not in those of colorectal cancer.

Recently, positive clinical studies on the effectiveness of pre-operative CRT on locally advanced rectal cancer have been reported (38). However, pre-operative CRT is not effective in all cases and, actually, cases in which no antineoplastic effect was obtained also exist. Since the treatment period for pre-operative CRT is approximately 10 weeks, patients who obtain no response to CRT lose valuable time during which they could have been treated more effectively. Thus, it is necessary to investigate factors which influence the efficacy of pre-operative CRT.

The results of the present study suggest that the presence of CD133 and CD24 expression is associated with the efficacy of pre-operative CRT. Assuming that CD133 and CD24 are predictive factors of the sensitivity to pre-operative CRT, patients with both $CD133^+$ and $CD24^+$ are expected to have low sensitivity to CRT. So, it may be recommended that such patients undergo surgery without first undergoing CRT. However, since patients with both $CD133^-$ and $CD24^-$ are expected to have high sensitivity to CRT, it may be necessary to aggressively treat these patients first with pre-operative CRT.

In conclusion, the present study shows that the overexpression of cancer stem cell-related factors, CD133 and CD24, is associated with the sensitivity of locally advanced rectal cancer to pre-operative CRT. Further prospective studies are required to establish a new therapeutic system that appropriately uses pre-operative CRT for the benefit of patients with locally advanced rectal cancer. Our group is presently conducting a prospective study using biopsy specimens from pre-therapeutic tumors (UMIN003398). This retrospective study provides valuable information for realization of the ongoing prospective study.

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