Elevated CD133, but not VEGF or EGFR, as a predictive marker of distant recurrence after preoperative chemoradiotherapy in rectal cancer

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Abstract. CD133 has been postulated to be a colon cancer stem cell (CSCs) marker. Recent investigations suggest that CSCs might contribute to cancer recurrence and resistance to conventional therapies. This study aimed to evaluate the role of CD133 in residual cancer cells after chemoradiotherapy (CRT) for rectal cancer. Forty patients with rectal cancer underwent CRT followed by surgery. Total RNAs of rectal cancer cells before (n=30) and after (n=40) CRT were isolated. Intratumoral CD133, vascular endothelial growth factor (VEGF), and epidermal growth factor receptor (EGFR) levels were measured using real-time reverse transcription polymerase chain reaction. Immunohistochemical staining of CD133 after CRT was also investigated. CD133 in residual cancer cells was higher than in stromal cells in post-CRT specimens ($p<0.0001$). The levels of CD133 were found to have increased in post-CRT specimens ($p=0.0184$), while VEGF and EGFR levels decreased during CRT ($p<0.0001$ and $p=0.0002$, respectively). Patients who developed distant recurrence had a higher post-CRT CD133 compared with those patients without recurrence ($p=0.0136$). Elevated post-CRT CD133 was associated with poor disease-free survival ($p=0.0168$). Immunohistochemical staining of the cytoplasmic and apical/endoluminal membranous CD133 was observed in residual cancer cells after CRT. CD133 expression in residual cancer cells after CRT may indicate a treatment resistant phenotype in putative CSCs. Elevated CD133, but not VEGF or EGFR, on FFPE specimens may be a predictive marker of distant recurrence and poor survival after preoperative CRT in rectal cancer.

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Abbreviations: VEGF, vascular endothelial growth factor; EGFR, epidermal growth factor receptor; RT-PCR, reverse transcription-polymerase chain reaction

Key words: CD133, vascular endothelial growth factor, epidermal growth factor receptor, chemoradiotherapy, rectal cancer

Introduction

Rectal cancer is one of the most common gastrointestinal cancers in Japan as well as in the Western world. Introduction of chemoradiotherapy (CRT) and total mesorectal excision (TME) has improved sphincter preservation rate, local pelvic control, and survival (1). Despite these significant improvements, distant recurrence remains the major cause of mortality in rectal cancer patients treated with preoperative CRT followed by TME (2).

A rare tumor subpopulation called cancer stem cells (CSCs) or tumor initiating cells (TICs) have been identified and proven in functional assays of stem/progenitor cell properties including the ability to self-renew, differentiate, and proliferate (3,4). CSCs appear to not only be responsible for tumorigenic capability but also confer resistance to chemotherapy or radiotherapy (5,6). Conventional cytotoxic therapies including CRT initially shrink the bulk of a tumor, but fail to eradicate it, resulting inevitably in cancer recurrence. Accordingly, we hypothesized that CRT decreased non-CSCs which are sensitive to CRT, while it may increase the percentage of putative CSCs characteristic of CRT resistance in the population of residual cancer cells.

CD133 (known as prominim-1) is regarded as one of the most important markers of colon CSCs by two studies reporting that CD133 expressing cells sorted by fluorescence-activated cell sorting system have shown tumor-forming abilities in xenografts (7,8). Pancreatic CSCs with CD133 expression demonstrated a higher metastatic potential compared with those without it (9). Stem cell-like glioma cells with CD133 expression promoted tumor angiogenesis

through the vascular endothelial growth factor (10). Accumulating evidence indicates that CSCs are associated with tumor metastasis and angiogenesis. Furthermore, CD133 has been reported to be associated with metastatic potential and poor survival in certain human malignancies (11,12).

Vascular endothelial growth factor (VEGF) is intimately involved in the regulation of tumor-associated angiogenesis and has been reported to be associated with tumor aggressiveness, metastasis and poor prognosis (13). Epidermal growth factor receptor (EGFR) is a cell membrane tyrosine kinase receptor which initiates a complex intracellular signal transduction cascade that promotes cancer cell division, migration, angiogenesis, and apoptosis inhibition. EGFR has been also reported to be associated with increased risk of metastasis and poor survival (14).

The VEGF and EGFR pathways have shown to be closely related to each other and share common downstream signaling. Recent preclinical and clinical studies demonstrate that combining VEGF and EGFR inhibition is a feasible therapeutic approach and provides promising efficacy in a range of solid tumors including colorectal cancer (15,16).

First, to clarify the potential association between CRT resistance and the existence of putative CSCs, CD133 expression was investigated in rectal cancer by using pre-CRT tumor biopsies and post-CRT micro-dissected residual cancer cells. Second, to examine the association between CD133 expression and tumor angiogenesis, correlation between CD133 and tumor angiogenesis-related VEGF and EGFR were also investigated during CRT. We also evaluated the association between post-CRT CD133 expression, clinicopathological variables, and survival.

Materials and methods

Patients. A total of 40 patients with locally advanced rectal cancer were included in the current analyses. All patients were treated with preoperative chemoradiotherapy (CRT) followed by surgery at the Department of Gastrointestinal and Pediatric Surgery in the Mie University Graduate School of Medicine. Selection criteria were the availability of the quality of isolated RNA for real-time PCR with complete clinical data.

Treatment. The treatment included pelvic radiotherapy using a four-field box technique and concomitant chemotherapy consisting of 5-FU given as 600 mg/m^2 administered intravenously for 24 h and UFT (Tegafur and Uracil) given as 400 mg/body weight administered orally for 5 days a week.

Preoperative radiotherapy was delivered to the whole pelvis at a dose of 20 Gy in 4 fractions (5 Gy per fraction) a week. All patients underwent computed tomography (CT) simulation for three-dimensional radiotherapy planning and were treated with a 10 MV photon using a linear accelerator. The radiation field encompassed a volume that included the primary tumor, mesorectum, presacral space, whole of the sacral hollow, and regional lymph nodes. The superior border was placed at L5/S1 and the inferior border at 3 cm or more caudal to the primary tumor. Rectal resection with TME was performed within 2 weeks of the end of CRT.

Pathological evaluation and treatment response. Pathological evaluation in the resection specimens was performed according to TNM classification (17). Tumor regression of the primary tumor was semi-quantitatively determined by the amount of viable tumor vs. the amount of fibrosis. This ranged from no evidence of any treatment effect to a complete response with no viable tumor identified, as described by Dworak *et al* (18). Tumor regression grade (TRG) 0 was defined as no regression; TRG 1, minor regression (dominant tumor with fibrosis in ≤25% of the tumor mass); TRG 2, moderate regression (dominant tumor with fibrosis in 26-50% of the tumor mass); TRG 3, good regression (>50% tumor regression); and TRG 4, total regression (no viable tumor cells, only fibrotic mass).

Microdissection in formalin-fixed, paraffin-embedded (FFPE) specimens. Tumor specimens were fixed in 10% formaldehyde solution and embedded in paraffin. Sections (10-μm thick) of FFPE specimens were stained with nuclear fast red and subsequently manually microdissected to collect residual cancer and stromal cells, with reference to hematoxylin and eosin sections.

RNA extraction from FFPE specimens. Microdissected samples were digested with proteinase K in lysis buffer containing Tris-HCl, EDTA, and sodium dodecyl sulfate as previously reported, with minor modifications (19). RNA was purified by phenol and chloroform extraction.

cDNA synthesis. cDNA was synthesized with random hexamer primers and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions.

Real-time quantitative RT-PCR. Real-time quantitative RT-PCR analysis was done with the SYBR-Green PCR Master Mix using an Applied Biosystems 7500 Real-Time PCR System according to the manufacturer's instructions (Applied Biosystems, Inc., Foster City, CA). Primers and probes for CD133, VEGF, EGFR and ß-actin were designed with Primer3 software (Biology Workbench Version 3.2, San Diego Supercomputer Center, at the University of California, San Diego, CA). Sequences are shown in Table I. PCR was performed in a final volume of 25 μl with a SYBR-Green PCR Master Mix using 1 μl cDNA, 400 nM of each primer for the respective genes. Cycling conditions were 50˚C for 2 min and 95˚C for 10 min followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min.

Relative mRNA levels of target genes. Relative mRNA levels were determined by the standard curve method. The standard curves and line equations were generated using 5-fold serially diluted solutions of cDNA from the colon cancer cell line LoVo. All standard curves were linear in the analyzed range with an acceptable correlation coefficient (R2). The level of target gene expression was calculated from the standard curve. Quantitative normalization of cDNA in each sample was performed using the expression of the ß-actin gene as an internal control. Finally, mRNA levels of the target gene were given as ratios to ß-actin mRNA levels. Real-

Table I. Primer and probe sequences of target genes.

Gene	Primer and probe	Sequence
CD133	Forward primer	5'-GCTTTGCAATCTCCCTGTTG-3'
	Reverse primer	5'-TTGATCCGGGTTCTTACCTG-3'
VEGF	Forward primer	5'-CAGAAGGAGGAGGGCAGAA-3'
	Reverse primer	5'-CTCGATTGGATGGCAGTAGC-3'
EGFR	Forward primer	5'-CCTATGTGCAGAGGAATTATGATCTTT-3'
	Reverse primer	5'-CCACTGTGTTGAGGGCAATG-3'
ß-actin	Forward primer	5'-ACAGAGCCTCGCCTTTGC-3'
	Reverse primer	5'-GCGGCGATATCATCATCC-3'

time PCR assays were done in duplicate for each sample and mean values were used for calculations of the mRNA levels.

Immunohistochemistry for CD133 in rectal cancer after CRT. Sections (2-μm thick) of FFPE specimens were made. After deparaffinization and dehydration for antigen unmasking, specimens were brought to a boil in 10 mM sodium citrate buffer. Specimens were then blocked and incubated with primary antibody overnight at 4˚C. The antibody was detected by Envision reagents (Envision kit/HRP, Dako Cytomation, Denmark). All sections were counterstained with hematoxylin. Primary CD133 rabbit monoclonal antibody (Cell Signaling Technology) was used in a dilution of 1:100 with labeled streptavidin-biotin method (LASB2 kit/HRP, Dako) added. Negative controls were run simultaneously with pre-immune immunoglobulin.

Statistical analysis. All statistical analyses were performed using JMP version 5 (SAS Institute Inc., Cary, NC, USA). The value of each target gene is expressed as median value (inter-quartile range).

Associations between gene expression levels (continuous variables) and clinicopathological variables (categorical variables) were evaluated using Mann-Whitney U test for two groups or Kruskal-Wallis test for multiple groups.

Disease-free survival was calculated from the date of surgery to the date of disease recurrence. Overall survival was calculated from the date of surgery to the date of death due to rectal cancer or last follow-up. Survival was evaluated using the Kaplan-Meier method. The log-rank test was used to compare the cumulative survival durations in the patient groups.

A non-parametric receiver operating characteristic (ROC) analysis was performed to calculate the best cut-off values predictive of distant recurrence using Medcalc 7.2 for Windows (Mariakerke, Belgium). P-values of <0.05 were considered statistically significant.

Results

Patient characteristics and survival. Forty patients were included in this study. The median age was 65 years (range 37-78 years); the male to female ratio was 3.4:1. The post-CRT pathological T stages were pT1 (10%), pT2 (25%), pT3 (62.5%), and pT4 (2.5%), respectively. Thirteen patients (32.5%) had pathological lymph node metastases. Lymphatic invasion was present in 31 of 40 patients (77.5%), and vascular invasion was present in 26 patients (65%), respectively. Thirty-four tumors (85%) showed well- or moderately-differentiated adenocarcinoma histology. None of the patients had local recurrence. Patterns of distant recurrence include liver and lung metastases in 2 patients, lung metastases alone in 3 patients, and peritoneal metastasis in one patient.

Medians of overall survival (OS) and disease-free survival (DFS) were 38.1 months (range; 6.8-86.3) and 41.3 months (range; 2.5-86.3), respectively. The presence of distant recurrence and pathological stage were significantly associated with OS ($p=0.0018$ and $p=0.0174$, respectively). Age over 65 years and pathological positive lymph nodes were significantly associated with DFS ($p=0.0486$ and $p=0.0469$, respectively). The other clinical parameters were not associated with OS or DFS.

Pathological response to chemoradiotherapy. The results of TRG are summarized in Table II. TRG was as follows: TRG 0, 0 patient (0%); TRG 1, 7 patients (17.5%); TRG 2, 17 patients (42.5%); TRG 3, 16 patients (40%) and TRG 4, 0 patient (0%). No TRG 4 (complete regression of the primary tumor) was seen in this study because of unavailability of residual cancer cells. Tumor or node downstaging was demonstrated in 23 patients (57.5%).

Correlations of CD133, VEGF and EGFR in pre- and post-CRT specimens. To clarify the role of CD133 expression in rectal cancer, correlations of CD133, VEGF and EGFR were examined in pre-CRT tumor biopsy specimens (n=30) and post-CRT FFPE specimens (n=40). There were significant positive correlations between CD133 and VEGF ($r=0.378$, $p=0.0392$), between CD133 and EGFR ($r=0.774$, $p<0.0001$) or between VEGF and EGFR ($r=0.390$ $p<0.0332$) in pre-CRT tumor biopsy specimens (Fig. 1). However, these correlations were not observed in post-CRT FFPE specimens (Fig. 2).

Table II. Association of post-CRT CD133, VEGF, and EGFR with clinicopathological variables.

Variable	No.	CD133	p-value	VEGF	p-value	EGFR	p-value
Gender							
Male	31	0.183		0.029		0.014	
Female	9	0.115	0.55	0.038	0.07	0.010	0.94
Age (median, 65 years)							
≤65	21	0.174		0.033		0.014	
>65	19	0.183	0.51	0.031	0.75	0.013	0.36
Pathological T category							
T1	4	0.123		0.039		0.027	
T2	10	0.119		0.027		0.012	
T3	25	0.219		0.031		0.012	
T4	1	0.278	0.22	0.092	0.78	0.021	0.52
Pathological N category							
Present	13	0.295		0.03		0.434	
Absent	27	0.139	0.32	0.032	0.59	0.274	0.90
Histology							
Well-moderate	34	0.179		0.032		0.015	
Poor/muc.	6	0.196	0.94	0.022	0.12	0.010	0.17
Lymphatic invasion							
Present	31	0.21		0.032		0.013	
Absent	9	0.107	0.32	0.029	1.00	0.012	0.53
Vascular invasion							
Present	26	0.286		0.032		0.013	
Absent	14	0.097	0.07	0.031	0.49	0.015	0.41
Tumor regression grading							
TRG 1	7	0.139		0.025		0.015	
TRG 2	17	0.183		0.03		0.014	
TRG 3	16	0.197	0.99	0.032	0.96	0.012	0.64

Expression of CD133, VEGF, and EGFR in matched pre- and post-CRT specimens. To confirm whether expression of CD133, VEGF, and EGFR were altered during CRT, the expression of each gene was compared between matched pre-CRT and post-CRT specimens (Table IV). CD133 was significantly increased in post-CRT specimens (p=0.0184), suggesting the selection of putative CSCs during CRT. In contrast, VEGF and EGFR were significantly decreased during CRT (p<0.0001 and p=0.0002, respectively).

Post-CRT CD133, VEGF, and EGFR levels in residual rectal cancer and stromal cells. Median value of post-CRT CD133 level was 0.179 (inter-quartile range, 0.051-0.563) in residual cancer cells, whereas it was only 0.003 (inter-quartile range, 0.001-0.009) in surrounding stromal cells. Post-CRT CD133 and VEGF were significantly higher in residual cancer cells compared with those in stromal cells (p<0.0001 and p=0.0002, respectively, Fig. 3). No significant difference in post-CRT EGFR levels were found between residual cancer and stromal cells.

Associations of post-CRT CD133, VEGF, and EGFR with clinicopathological variables. Median value of post-CRT CD133, VEGF and EGFR mRNA levels were 0.179 (inter-quartile range; 0.051-0.563), 0.032 (0.018-0.040), and 0.013 (0.009-0.020), respectively.

Associations between post-CRT CD133, VEGF and EGFR and clinicopathological variables are summarized in Table II. No significant associations of post-CRT CD133, VEGF and EGFR with clinicopathological variables were observed.

As shown in Table III, patients who developed distant recurrence (n=6) had significantly higher post-CRT CD133 levels compared with those patients without recurrence (n=34) (p=0.0136). No significant association between distant recurrence and VEGF or EGFR was observed.

Predictive value of post-CRT CD133 for distant recurrence and disease-free survival. On the basis of these results, receiver operating characteristic curve (ROC) analyses were

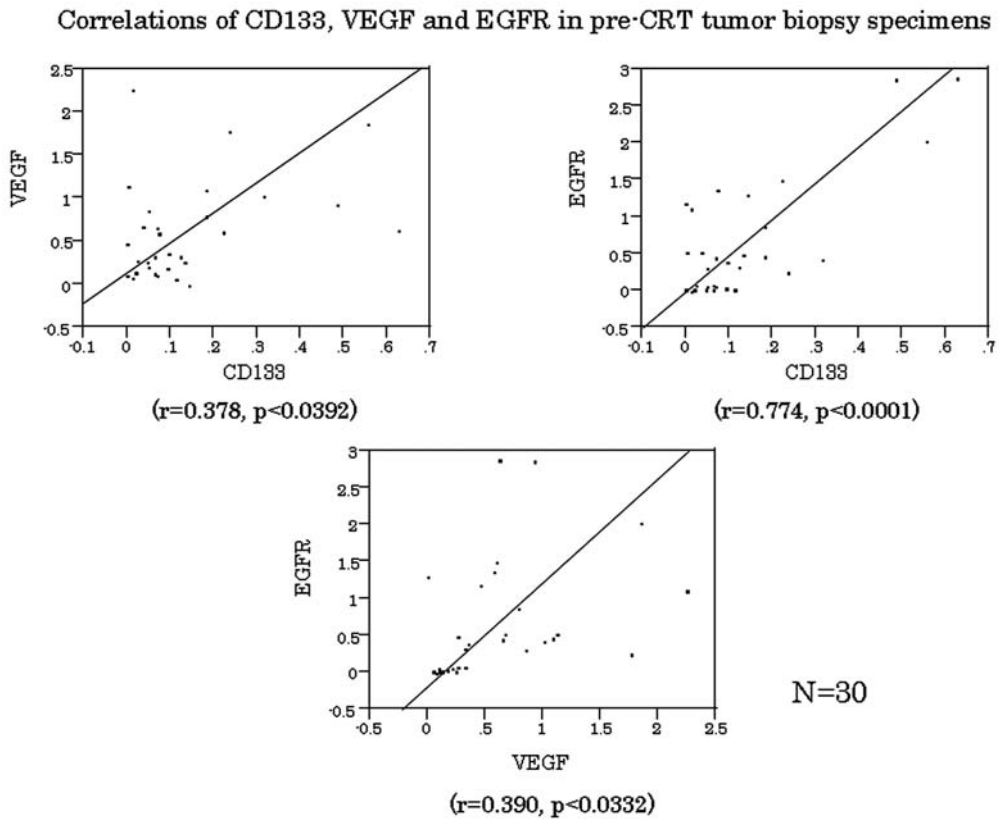

Figure 1. Correlations of CD133, VEGF and EGFR in pre-CRT tumor biopsy specimens. There were significant positive correlations between CD133 and VEGF (r=0.378, p=0.0392), between CD133 and EGFR (r=0.774, p<0.0001) or between VEGF and EGFR (r=0.390 p<0.0332) in pre-CRT tumor biopsy specimens.

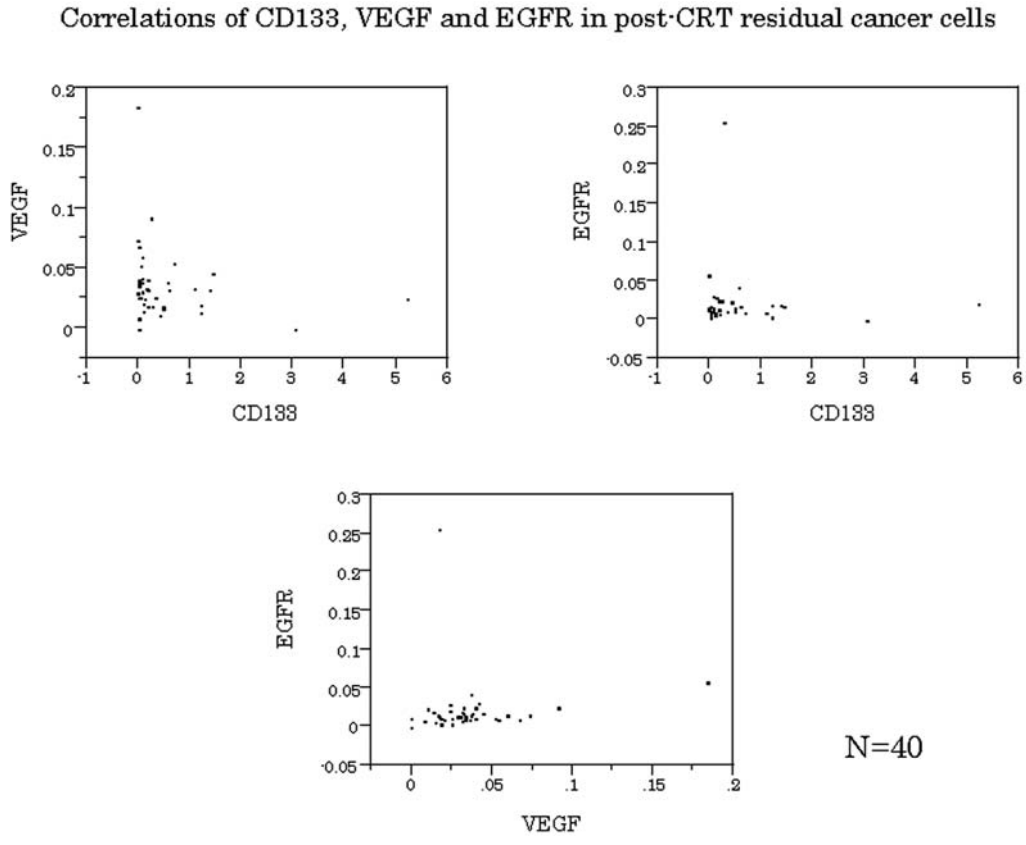

Figure 2. Correlations of CD133, VEGF and EGFR in post-CRT residual cancer cells. No significant correlations were observed in post-CRT FFPE specimens.

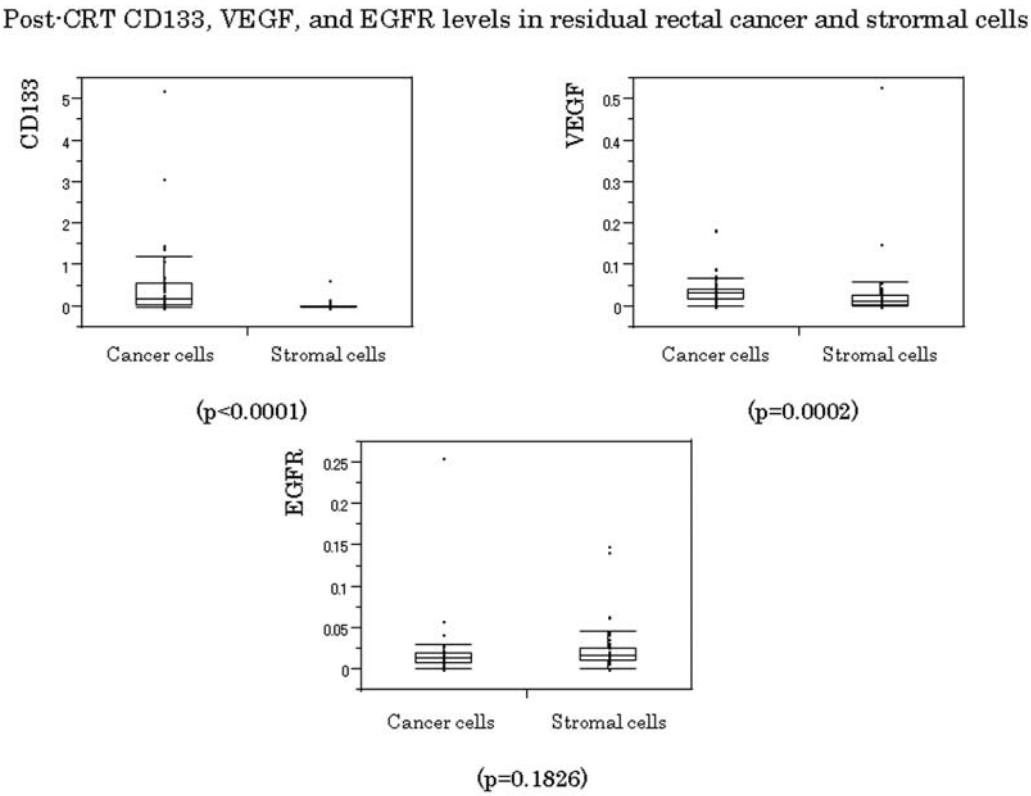

Figure 3. Post-CRT CD133, VEGF, and EGFR levels in residual rectal cancer and stromal cells. Post-CRT CD133 was significantly higher in residual cancer cells compared with those in stromal cells (p<0.0001). Post-CRT VEGF was also higher in residual cancer cells compared with those in stromal cells (p=0.0002). No significant difference in post-CRT EGFR levels was found between residual cancer and stromal cells. Box and whisker plots were used to summarize the distribution of mRNA level in residual cancer and stromal cells in post-CRT specimens. The horizontal line in the box represents the 50th (median) and the upper and lower lines of the box represent 75th and 25th quartiles, respectively. The whiskers indicate the range of the measurements.

Table III. Association of post-CRT CD133, VEGF, and EGFR with distant recurrence.

Gene of target	Patients with distant recurrence (n=6)	Patients without distant recurrence (n=34)	p-value
CD133	0.833 (0.048-2.143)	0.156 (0.0481-0.500)	**0.02**
VEGF	0.035 (0.017-0.056)	0.032 (0.018-0.039)	0.97
EGFR	0.013 (0.008-0.021)	0.013 (0.009-0.021)	0.6

Values of each target gene are expressed as median value (inter-quartile range). Bold type indicates a significant value.

Table IV. Expression of CD133, VEGF, and EGFR in matched pre-and post-CRT specimens.

Gene of targets	Pre-CRT specimen (n=30)	Post-CRT specimens (n=30)	p-value
CD133	0.074 (0.035-0.185)	0.191 (0.079-0.803)	**0.0184**
VEGF	0.413 (0.172-0.875)	0.032 (0.018-0.043)	**<0.0001**
EGFR	0.404 (0.047-1.128)	0.014 (0.010-0.021)	**0.0002**

Values of each target gene are expressed as median value (inter-quartile range). Bold type indicates a significant value.

used to identify each cut-off value of CD133 predictive of distant recurrence. A non-parametric ROC analysis showed that the best cut-off values of CD133 was 10.26. As shown in Fig. 4, patients with post-CRT CD133 above cut-off

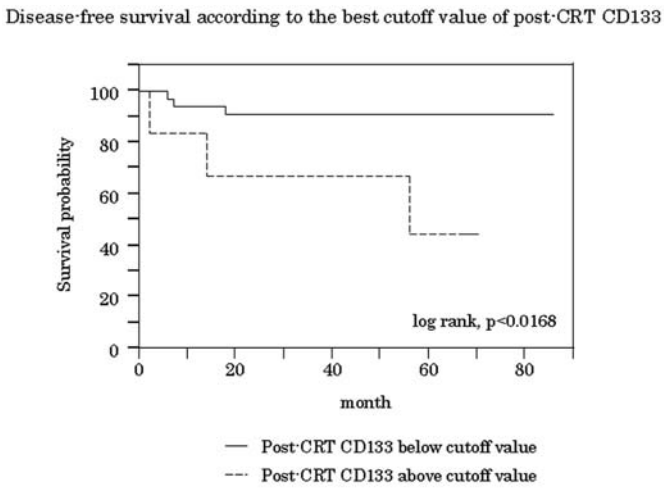

Figure 4. Disease-free survival according to the best cut-off value of post-CRT CD133. Disease-free survival was evaluated using the Kaplan-Meier method. The log-rank test was used to compare the cumulative survival durations in the patient groups. Patients with post-CRT CD133 above cut-off value showed significantly worse disease-free survival ($p<0.0168$).

value showed significantly worse disease-free survival ($p<0.0168$).

Immunohistochemistry for CD133 in residual cancer cells after CRT. The minority of residual cancer cells had immunoreactive CD133 protein expression within entire residual tumor (Fig. 5).

CD133 was observed diffusely in cytoplasm of residual cancer cells and was also located at the apical/endoluminal surface (membranous) or cytoplasm (cytoplasmic) or both of residual cancer cells (Fig. 6).

Discussion

We showed the correlations between CD133 and angiogenesis-related genes such as VEGF and EGFR during CRT in rectal cancer. Positive correlations of CD133, VEGF and EGFR in pre-CRT rectal cancer were observed, but these correlations were not observed in post-CRT residual cancer cells. CD133 levels increased after CRT on paired specimens of each patient. In contrast, both VEGF and EGFR levels decreased after CRT.

There are several interpretations of these results. First of all, CRT may change these gene expression levels, resulting in the loss of correlations after CRT. Second, CRT may cause an imbalance between two putative populations within the tumor. A majority of tumor cells expressing VEGF and EGFR (considered as non-CSCs) may respond to CRT and subsequently shrink or disappear. In contrast, a very small population of cells expressing CD133 (considered as CSCs) may resist CRT and be left as residual cancer cells on post-CRT specimens. Third, decreased expression levels of VEGF and EGFR may be partly due to the normalization of tumor hypoxia by CRT.

Although several factors may influence expression of CD133, VEGF, and EGFR during CRT, CD133 seems to be associated with tumor angiogenesis through VEGF or

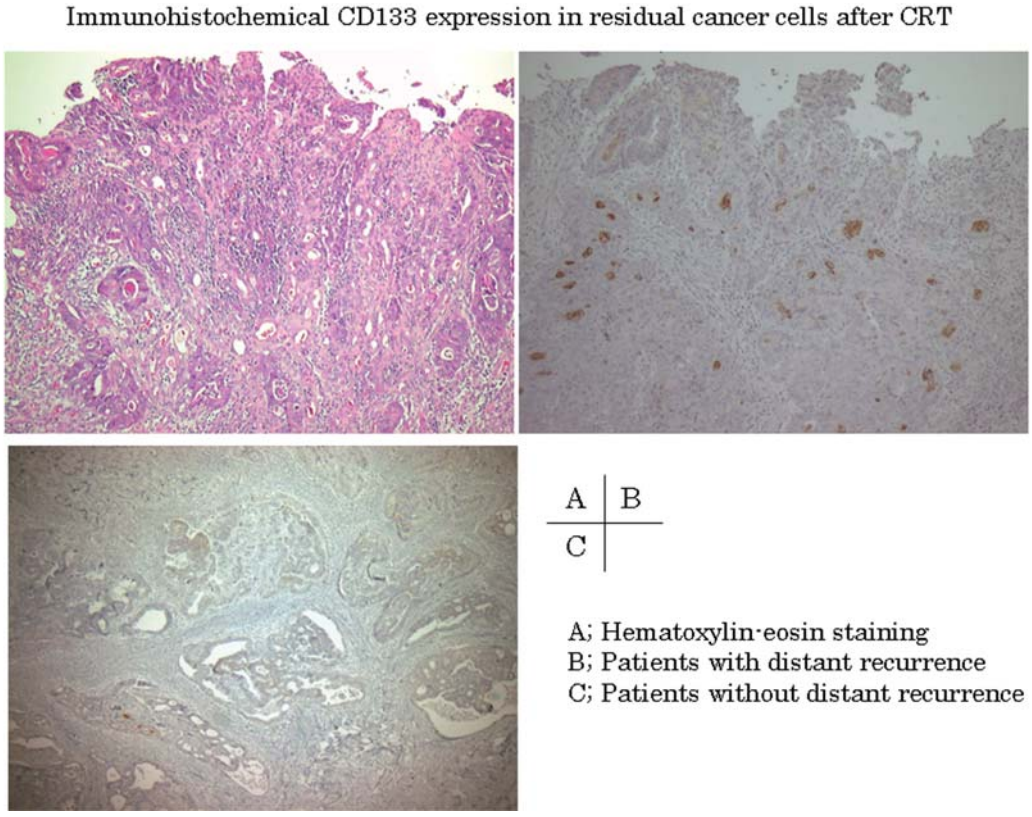

Figure 5. Immunohistochemical CD133 expression in residual cancer cells after CRT. The minority of residual cancer cells had immunoreactive CD133 protein expression within the entire residual tumor.

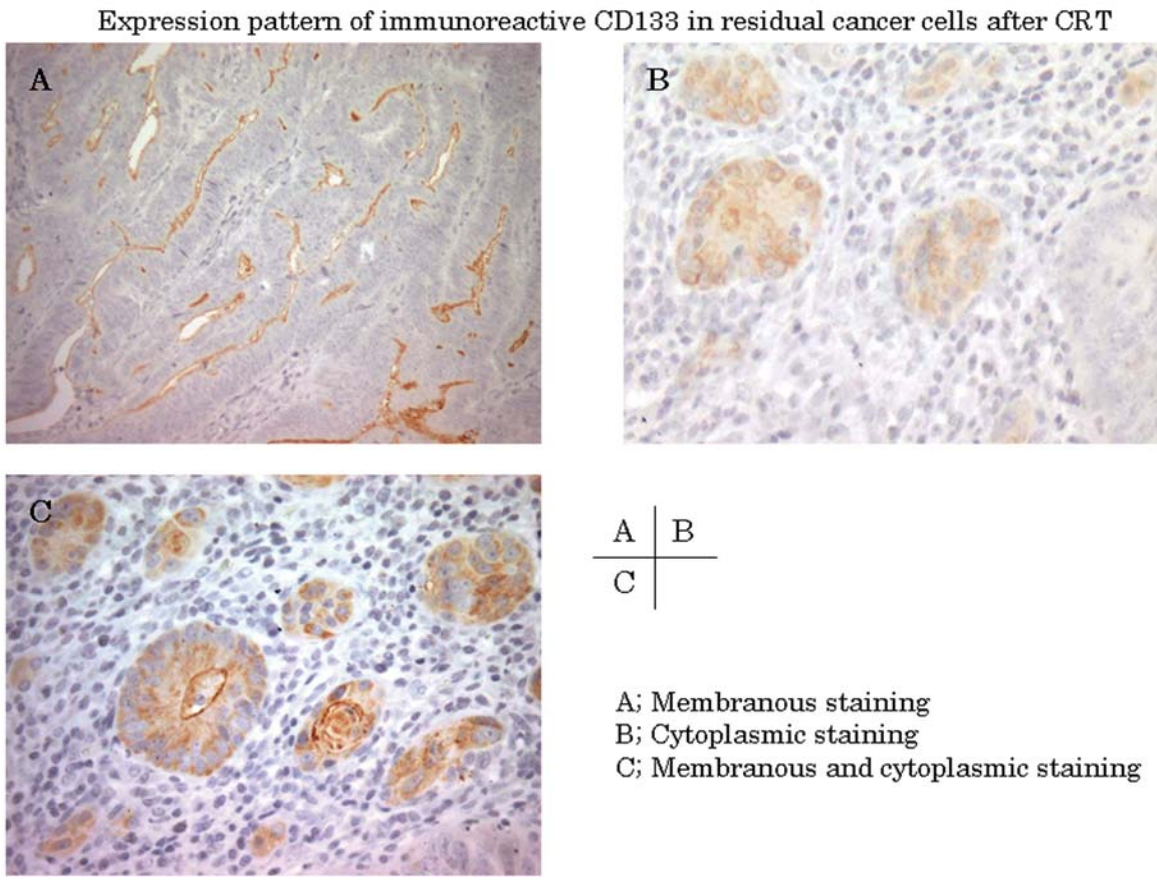

Figure 6. Expression pattern of immunoreactive CD133 in residual cancer cells after CRT. CD133 was observed at the apical/endoluminal surface (A) or in cytoplasm (B) or both (C) of residual cancer cells.

EGFR. We also demonstrated that post-CRT CD133 was associated with distant recurrence and poor disease-free survival in rectal cancer, although we failed to provide any prognostic value of CD133 on pre-CRT specimens. Recently, the prognostic role of CD133 has been reported in pancreatic (11) and colorectal cancer (12). Prognostic value of TRG has been also reported in rectal cancer patients after CRT followed by TME (20). TRG is based on the percentage of residual cancer cells to stroma of entire tumor beds after CRT. The amount of residual cancer cells after CRT seems to be predictive of disease recurrence and survival in relation to CRT resistance. Our result showing the association of post-CRT CD133 with distant recurrence and poor survival appears to be consistent with a previous report on prognostic value of TRG.

Immunostaining of CD133 in residual cancer cells after CRT was shown in this study. CD133 positivity or immunohistochemical evaluation for CD133 remains controversial (11,12,21). Recent studies propose that apical/endoluminal membranous CD133 staining was characteristic of well-oriented, polarized and differentiated cells, while cytoplasmic CD133 staining was found in minor population of cells, suggesting that cancer cells with cytoplasmic CD133 staining may be indicative of putative CSCs (21,22). Residual cancer cells represented membranous, cytoplasmic, or both of CD133 staining. As shown in Fig. 6, not all residual cancer cells expressed CD133. It is possible that post-CRT residual cancer cells may have relatively higher percentage of putative CSCs with CD133 expression. Post-CRT CD133 immunostaining may be useful in identifying rectal cancer patients with increased risk of distant recurrence after CRT if reliable and reproducible evaluation criteria for immunoreactive CD133 protein would be established.

However, data from this study should be interpreted with caution. The main limitation of this study was the relatively small number of patients (n=40), including only six patients with distant recurrence. Large prospective trials will be needed to confirm the validity of the predictive value of post-CRT CD133 for distant recurrence.

In conclusion, CD133 in residual cancer cells after CRT may indicate a treatment resistant phenotype in putative CSCs. Elevated CD133, but not VEGF or EGFR, on FFPE specimens may be a predictive marker of distant recurrence and poor survival after preoperative CRT in rectal cancer. Expression analysis of CD133 in residual cancer may be useful for treatment stratification and clinical management in rectal cancer patients.

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