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2

Clinical Significance of Putative Cancer Stem Cells in Residual Cancer Cells After Chemoradiotherapy for Rectal Cancer

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1. Introduction

Cancer stem cells (CSC) seem to be resistant to conventional chemo- and radiation therapies when compared with non-CSCs. Conventional cytotoxic therapies initially shrink the bulk of a tumor, but fail to eradicate it, resulting in tumor recurrence. Treatment failure may in part be due to the resistance of CSCs to chemotherapy or radiotherapy (Baumann et al., 2008; Eyler & Rich, 2008).

Drug or radiation surviving cells (residual tumor cells following treatment) have been shown to contain a higher frequency of putative CSCs in a number of human malignancies. Bao et al. demonstrated that the population of cells enriched for glioma CSCs was dramatically increased by irradiation and that radioresistant gliomas showed an increased percentage of CD133 positive cells (Bao et al., 2006). Tsuchida et al showed that anti-cancer drug treatment increases the side-population fraction (considered CSCs) in cancer cell lines (Tsuchida et al., 2008).

Over the past decades, preoperative chemoradiotherapy (CRT) has been established as one approach in the multimodal treatment of several types of gastrointestinal malignancies. In rectal cancer, preoperative CRT followed by surgery has improved sphincter preservation, local pelvic control and survival of patients with locally advanced rectal cancer (Bosset et al., 2006; Guillem et al., 2005). However, disease recurrence (especially for distant metastases) remains the major cause of mortality in these patients (Collette et al., 2007; van den Brink et al., 2004).

In rectal cancer, tumor regression grading (TRG) following CRT was determined by quantifying the proportion of residual cancer cells to the stroma of the entire tumor bed on formalin-fixed paraffin embedded (FFPE) specimens. TRG or pathologic response has been shown to predict clinical outcome (disease recurrence or patient survival) of oesophageal (Brücher et al., 2006; Chirieac et al., 2005), gastric (Patel et al., 2007; Rohatgi et al., 2006), or rectal cancer (Rödel et al., 2005) in patients after preoperative CRT followed by surgery, rather than pre-CRT clinical stage. The amount of residual cancer cells after CRT seems to be predictive of disease recurrence and survival in relation to CRT resistance.

Therefore, we hypothesized that CRT decreased or eradicated non-CSCs, which are sensitive to CRT, while increasing the percentage of putative CSCs characteristic of CRT resistance in the population of residual cancer cells. Residual cancer cells following CRT may be expected to contain a higher frequency of putative CSCs expressing stem cell markers, compared to primary, non-CRT tumor cells.

To test this hypothesis, we investigated the expression of stem cell markers in post-CRT residual cancer cells on FFPE specimens using microdissection and real-time quantitative reverse transcription polymerase chain reaction (RT-PCR).

2. Microdissection and RNA isolation from FFPE specimens

2.1 Microdissection in FFPE specimens

Tumor specimens were fixed in 10% formaldehyde solution v/v and embedded in paraffin. FFPE specimens (10 μm sections) were stained with nuclear fast red (Vector Laboratories, Inc., CA) and subsequently manually microdissected under microscope magnification (from ×5 to ×10). Residual cancer cells were isolated using a sterile blade and carefully collected with reference to hematoxylin and eosin sections, containing more than 70% cancer cells. Fibrotic tissue areas, necrotic cells, and non-neoplastic cells were identified.

2.2 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 modification (Bijwaard et al., 2001). RNA was purified using phenol and chloroform extraction. Isolated RNA was purified using ethanol precipitation. The concentration and quality of RNA was measured with UV absorbance at 260 nm and 280 nm (A260/280 ratio).

3. Expression of stem cell markers in residual cancer cells after CRT

3.1 cDNA synthesis

To reverse transcribe the fragmented mRNA from FFPE tissue materials, we used random hexamer priming, instead of oligo (dT)-based priming. cDNA was synthesized with random hexamer and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

3.2 Real-time quantitative RT-PCR

Real-time quantitative RT-PCR analysis was performed using a fluorescence-based real-time detection method (TaqMan) and an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Inc., Foster City, CA). Although SYBR-Green based detection is less specific than TaqMan-based detection, we used SYBR-Green based detection to save experimental time and costs.

Primers were strictly selected or designed to be intron spanning to avoid amplification from contaminated genomic DNA. Target sequences were kept as small as possible (approximately 100 bp) to ensure the detection of fragmented and partially degraded RNA. To confirm primer specificity, a single band of expected amplicon size for each target gene was verified using gel electrophoresis on a 2% agarose gel and visualized with ethidium bromide.

Primers for CD133, CD44, OCT4, SOX2, VEGF, and beta actin were designed with primer3 software (Biology Workbench Version 3.2, San Diego Supercomputer Center, at the University of California, San Diego). Primers for EGFR were synthesized according to previously published sequences (Schneider et al., 2005). Primer sequences are shown in Table 1. PCR was performed in a final volume of 25 µl with a SYBR Green PCR Master Mix using 1 µl cDNA, and 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 s and 60°C for 1 min.

gene	primer	sequence	product size
CD133	Forward	5'-GCTTTGCAATCTCCCTGTTG-3'	
	Reverse	5'-TTGATCCGGGTTCTTACCTG-3'	94bp
CD44	Forward	5'-CGGACACCATGGACAAGTTT-3'	
	Reverse	5'-CACGTGGAATACACCTGCAA-3'	115bp
OCT-4	Forward	5'-CTGGAGAAGGAGAAGCTGGA-3'	
	Reverse	5'-CAAATTGCTCGAGTTCTTTCTG-3'	79bp
SOX2	Forward	5'-CAAGATGCACAACTCGGAGA-3'	
	Reverse	5'-GCTTAGCCTCGTCGATGAAC-3'	95bp
VEGF	Forward	5'-CAGAAGGAGGAGGGCAGAA-3'	
	Reverse	5'-CTCGATTGGATGGCAGTAGC-3'	80bp
EGFR	Forward	5'-CCTATGTGCAGAGGAATTATGATCTTT-3'	
	Reverse	5'-CCACTGTGTTGAGGGCAATG-3'	88bp
Beta actin	Forward	5'-ACAGAGCCTCGCCTTTGC-3'	
	Reverse	5'-GCGGCGATATCATCATCC-3'	75bp

Table 1. Primer sequences of target genes

3.3 Relative mRNA levels of target genes

The parameter Ct (threshold cycle) is defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe passes a fixed threshold above baseline. The Ct is inversely proportional to the amount of cDNA, i.e., a higher Ct value means that more PCR cycles are required to reach a certain level of detection.

Relative mRNA levels were determined by the standard curve method. Standard curves and line equations were generated using five-fold serially diluted solutions of cDNA from colon cancer cell line, Lovo. All standard curves were linear in the analyzed range with an acceptable correlation coefficient (R^2). Target gene expression was calculated using the standard curve.

Quantitative normalization of cDNA in each sample was performed using the expression of the beta actin gene as an internal control. Finally, mRNA levels of the target gene were presented as ratios between the genes of interest and the internal reference gene (beta actin). Real-time PCR assays were performed twice for each sample and mean values were used for calculations of mRNA levels.

4. Difference in gene expression profile of primary tumor and residual tumor following CRT

4.1 Correlations of CD133, SOX2 and OCT4 mRNA levels in pre-CRT or post-CRT tumor cells

A cell surface protein CD133, known as prominim-1, has been regarded as one of the most important markers of colorectal CSCs (O'Brien et al., 2007; Ricci-Vitiani et al., 2007). OCT4 and SOX2 are essential transcription factors for normal pluripotent cell development and maintenance in embryonic stem (ES) cells, which are also known as reprogramming genes that induce an ES cell-like state in fibroblasts i.e., induced pluripotent stem (iPS) cells (Takahashi et al., 2007; Yamanaka, 2008).

As shown in Fig.1-1, we examined how these 'stem cell' related genes were correlated in primary non-treated tumor cells and post-CRT residual tumor cells. A positive correlation between OCT4 and SOX2 was observed in pre-CRT endoscopic tumor specimens. There was no correlation between CD133 and OCT4 or SOX2 in pre-CRT specimens. In post-CRT residual cancer on FFPE specimens (Fig. 1-2), significant positive correlations among all three stem cell markers were seen (Saigusa et al., 2009).

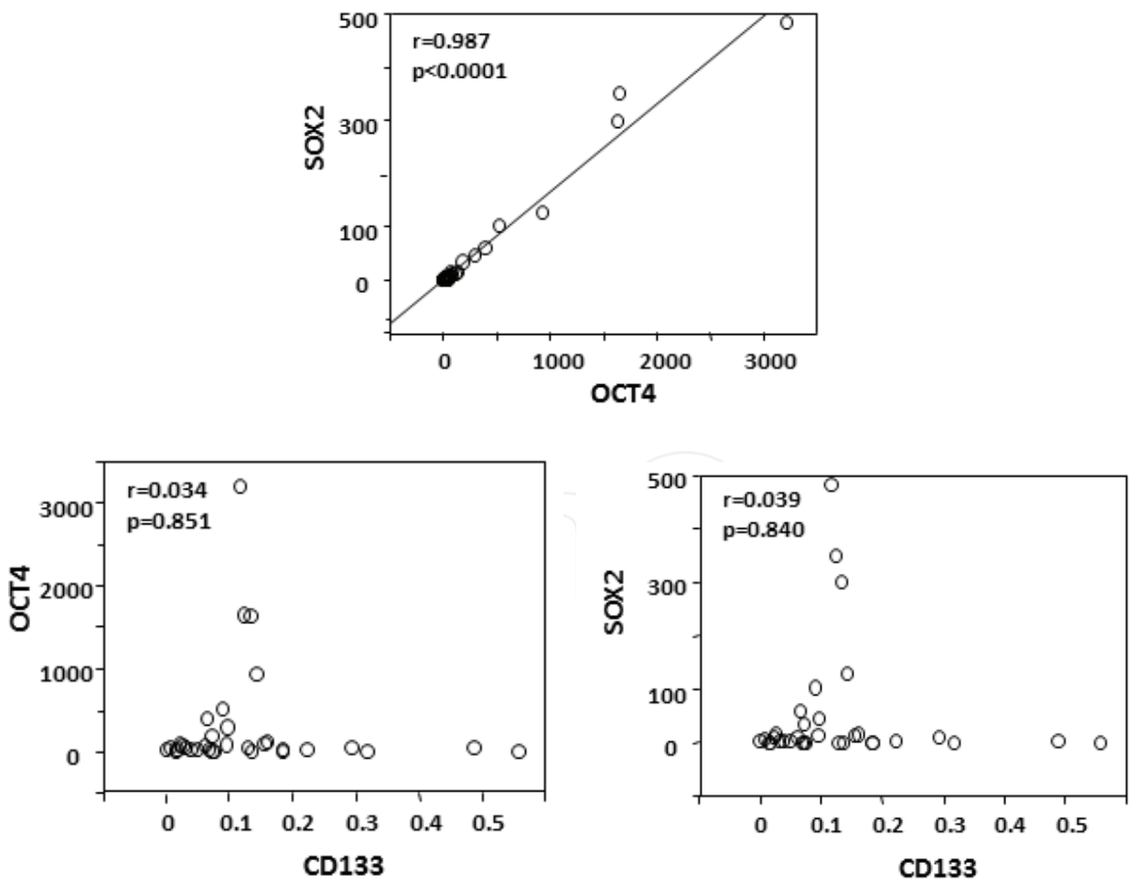

Fig. 1-1 Correlation between CD133, SOX2 and OCT4 in pre-CRT tumor biopsy specimens

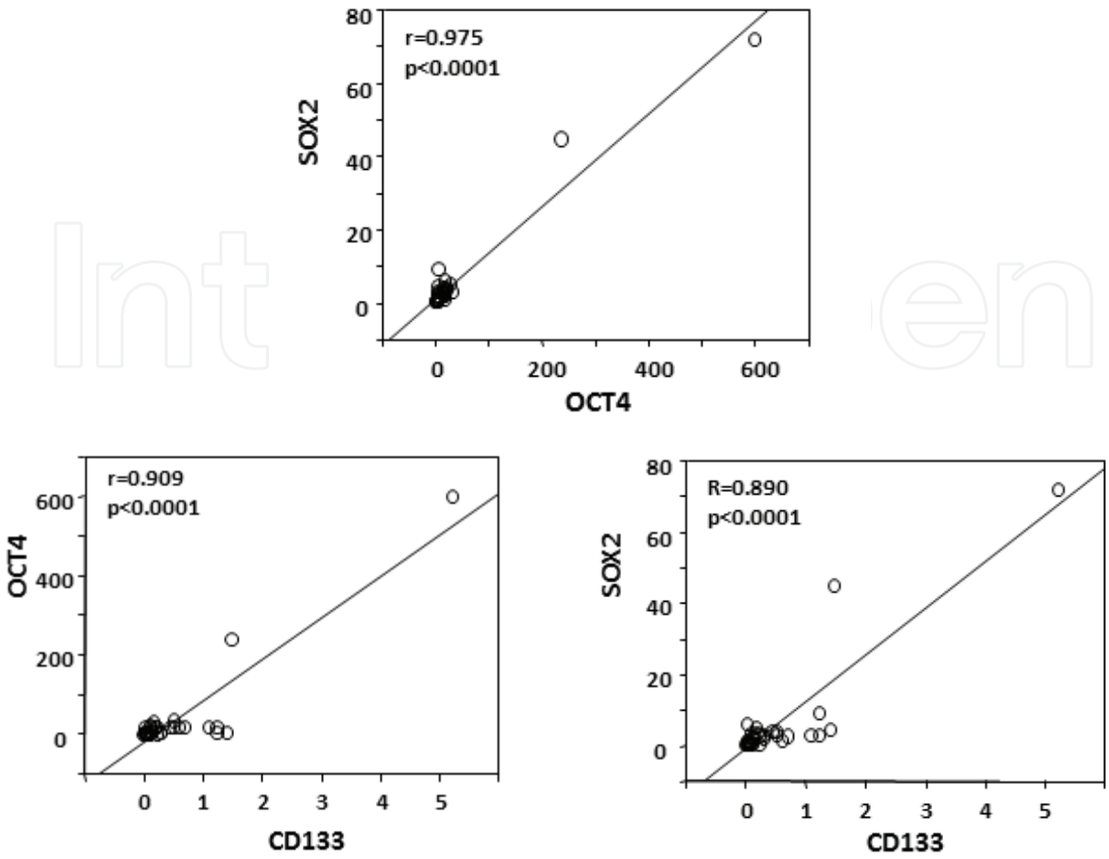

Fig. 1-2 Correlation between CD133, SOX2 and OCT4 in post-CRT residual cancer cells

Strong co-expression of OCT4 and SOX2 in both pre-and post-CRT tumor cells may indicate that these two genes have an indistinguishable relationship associated with maintenance of pluripotency in stem cells.
Also, residual cancer cells surviving CRT may enrich a population of putative CSCs expressing CD133, OCT4 and SOX2 because of the potential association between CSCs and treatment resistance.

4.2 Correlations of CD133, VEGF and EGFR mRNA levels in pre-CRT or post-CRT tumor cells

The vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) pathways are closely associated with each other, and share common downstream signaling, especially for tumor angiogenesis. Increased expression of VEGF or EGFR has been reported to be involved in tumor aggressiveness, metastasis, and poor prognosis in several types of malignancies (Galizia et al., 2004; Galizia et al., 2006). To date, anti-VEGF and anti-EGFR antibodies have become indispensible in the treatment of metastatic CRC (Chau & Cunningham, 2009). In other words, both VEGF and EGFR are important therapeutic targets in CRC.
We then examined how CD133 correlated with these therapeutic targets for CRC in primary tumor and post-CRT residual tumor cells. There were significant positive correlations between CD133 and VEGF, between CD133 and EGFR or between VEGF and EGFR in pre-

CRT tumor biopsy specimens (Fig. 2-1). However, these correlations were not observed in post-CRT FFPE specimens (Fig. 2-2; Yasuda et al., 2009).
We previously described several possibilities for explaining these findings. We believe it seems plausible that CRT may cause an imbalance between two distinct populations (putative CSCs and non-CSCs) within the tumor. A majority of tumor cells expressing VEGF and EGFR (considered as non-CSCs) may respond to CRT and then shrink or disappear. By contrast, a very small population of tumor cells expressing CD133 (considered as CSCs) may resist CRT and be left as residual cancer cells in post-CRT specimens.

4.3 CD133 and CD44 expression in pre-CRT or post-CRT tumor cells

CD44 is a transmembrane glycoprotein molecule, which is widely expressed as a cell surface hyaluronan receptor in normal epithelial, mesenchymal and hematopoietic cells. Also, CD44 has been reported as one of the important cell surface markers for isolating colorectal CSCs (Du et al., 2008; Haraguchi et al., 2008).
We examined whether the expression of potential markers (CD133 and CD44) for colorectal CSCs were changed during CRT. As shown in Fig. 3, tumoral CD133 mRNA levels were significantly increased in post-CRT resected specimens, compared with pre-CRT biopsy specimens. By contrast, tumoral CD44 mRNA levels were significantly decreased in residual cancer cells from post-CRT resected specimens (Yasuda H et al., 2009).

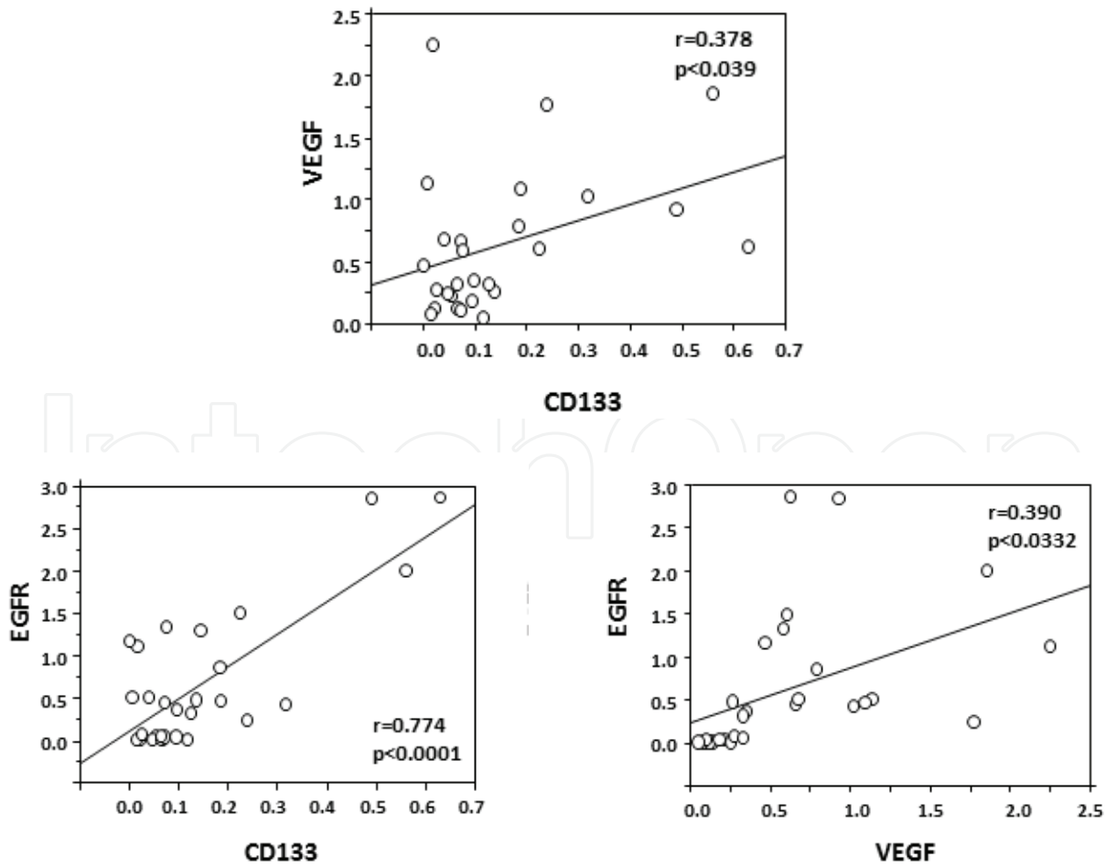

Fig. 2-1 Correlation between CD133, VEGF and EGFR in pre-CRT tumor biopsy specimens

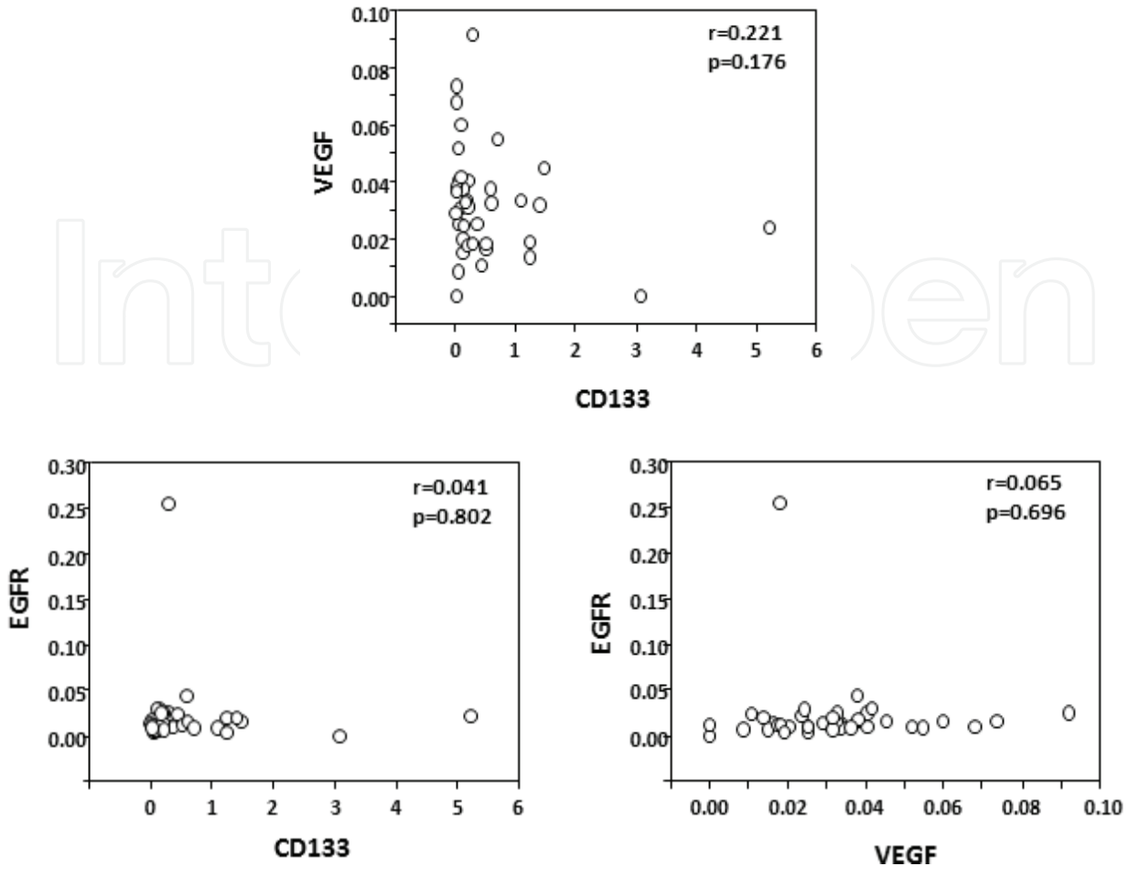

Fig. 2-2 Correlation between CD133, VEGF and EGFR in post-CRT residual cancer cells

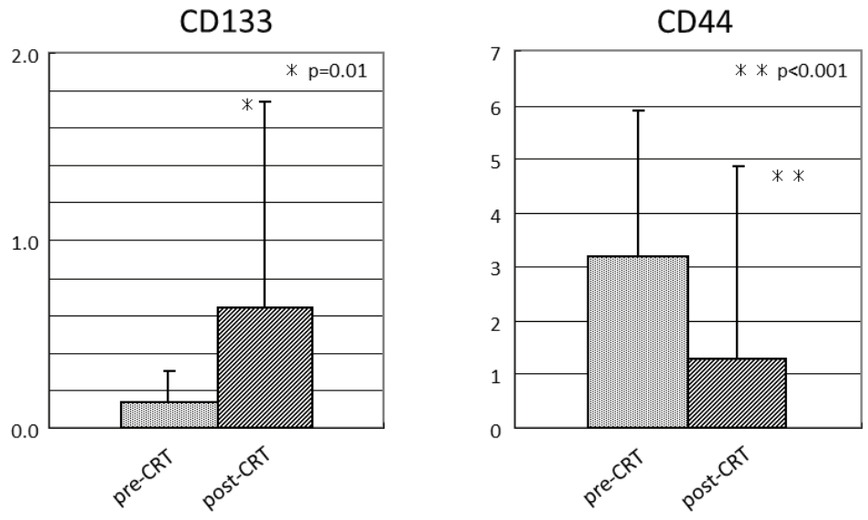

Fig. 3. CD133 and CD44 expression in paired pre-and post-CRT specimens

The comparison of potential surface markers for colorectal CSCs between pre-CRT and post-CRT tumor cells indicate that changes in expression of CD133 and CD44 during CRT were

quite opposite. We speculate that residual cancer following CRT may contain more CSCs than primary tumors before CRT. The relative proportion of CSCs may increase in residual cancer following CRT. Thus, gene expression related to CSCs may also increase in residual cancer following CRT, compared with primary tumors. In this context, CD133 seemed preferable to CD44 as the marker for colorectal CSCs.

5. Radiation surviving cells *in vitro* and residual cancer cells after CRT

5.1 CD133 and CD44 in radiation surviving HT-29 cells *in vitro*

We performed an *in vitro* experiment using human colorectal cancer cell lines to determine whether irradiation itself can induce the expression of CD133 or CD44.

Exponentially growing colorectal cells of the HT29 cell line were plated on a 10 cm dish and irradiated at a dose of 1, 2.5, and 5 Gy (CAX-150-20, Chubu medical Co. Ltd). Fourteen days later, colony formation assays were performed to evaluate cell survival after irradiation. Approximately 23%, 7%, and 5% survival fraction were found following irradiation with 1.0, 2.5, and 5.0 Gy, respectively. These surviving cells were collected for western blotting analysis. Single dose of 2.5 Gy and 5 Gy radiation increased CD133 protein levels, compared with control (Fig. 4-1). Densitometric analysis showed that CD133 was 1.4 times increased at 5 Gy radiation with respect to control. By contrast, radiation decreased CD44 protein levels regardless of radiation dose.

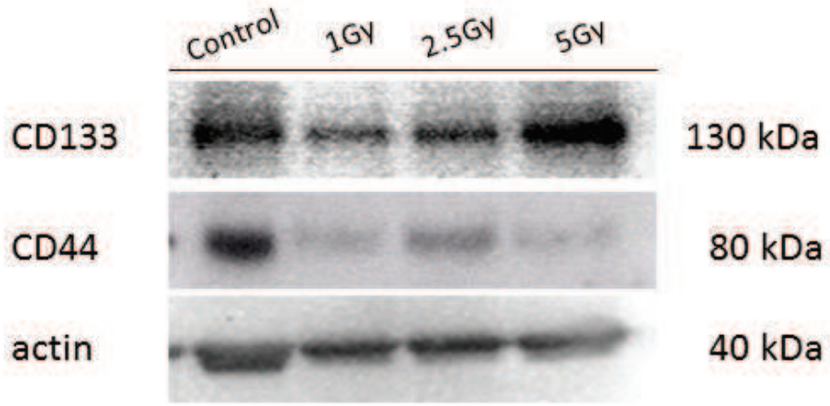

Fig. 4-1 CD133 and CD44 in radiation surviving HT-29 cells *in vitro*

CD133 increased in a radiation-dose dependent manner, despite the decreased number of radiation-surviving HT-29 cells. These *in vitro* results were consistent with CD133 mRNA levels increasing in residual cancer cells after CRT, compared with primary tumor cells before CRT. These results suggest that CRT may enrich the relative proportion of CD133 expressing CSCs within residual cancer, or that CRT may induce the expression of CD133 in tumor cells, or both.

5.2 Immunoreactive CD133 and CD44 in residual cancer cells after CRT

For immunohistochemical analysis, CD133 rabbit monoclonal antibody (Cell Signaling Technology, Inc. Boston, MA) and CD44 mouse monoclonal antibody (R&D Systems, Inc. Minneapolis, MN) were used. The primary antibody was detected using Envision reagents (Envision kit/HRP, Dako Cytomation, Denmark).

Immunoreactive CD133 and CD44 expression were observed in the minority of residual cancer cells within entire residual tumors (Fig. 4-2). There was no obvious concordance between CD133 and CD44 positivity of residual cancer cells, which may support the notion that CD133 positive and CD44 positive cells did not colocalize in colorectal cancer specimens (Du et al., 2008).

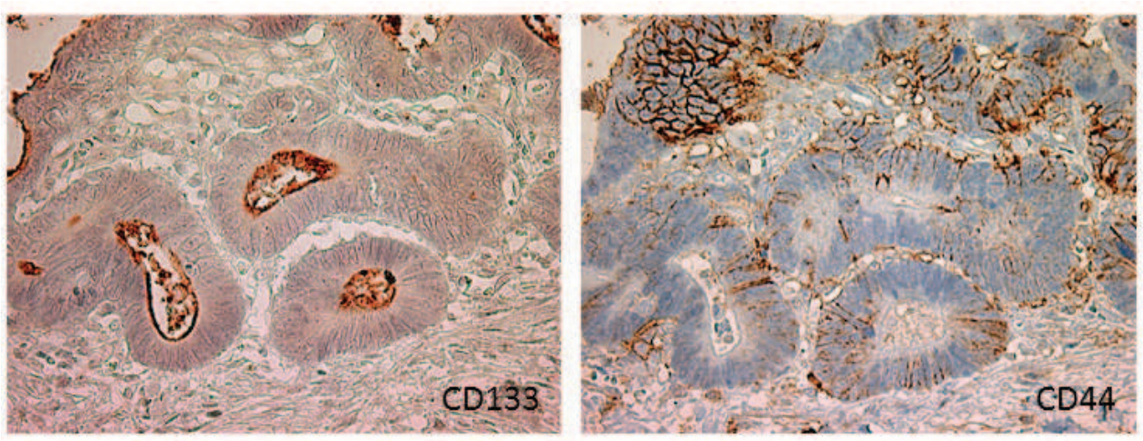

Fig. 4-2 Immunoreactive CD133 and CD44 in residual cancer cells after CRT

CD133 immunostaining in residual cancer cells after CRT showed not only apical/endoluminal membranous staining, but also cytoplasmic staining. Immervoll et al reported that apical/endoluminal membranous CD133 staining was characteristic by well-oriented, polarized and differentiated cells, while cytoplasmic CD133 staining was found in a minor population of cells (Immervoll et al., 2008). We have previously reported that residual cancer cells after CRT showed strong CD133 and moderate OCT4 and SOX2 staining, but no CK20 staining (a known epithelial marker) was observed (Saigusa et al., 2009). These lines of evidence suggest that CRT may induce dedifferentiation of cancer cells or may select putative CSCs with undifferentiated phenotype.

To determine if CRT may increase the relative proportion of CD133 expressing CSCs within residual cancer, it is necessary to compare the number of CD133 expressing tumor cells in pre-CRT endoscopic biopsy specimens and post-CRT resected specimens. This study has not yet been completed.

6. Clinical significance of CD133, OCT4, and SOX2 expression on residual cancer cells in patients with rectal cancer

6.1 Association of post-CRT CD133, CD44, OCT4, and SOX2 expression with clinicopathological variables

Thirty-three patients undergoing CRT followed by surgery were analyzed for an association between post-CRT OCT4 and SOX2 expression with clinical outcome. A total of 52 patients were analyzed for an association of post-CRT CD133 and CD44 expression with clinical outcome.

Patients who developed distant metastatic recurrence (e.g. liver, lung) had a significantly higher post-CRT CD133, OCT4, and SOX2 compared with those patients without recurrence. No such relationship was observed for post-CRT CD44.

6.2 Association of post-CRT CD133, CD44, OCT4, and SOX2 expression with patient survival

To identify the cut-off values of CD133, CD44, OCT4, and SOX2 predictive of distant metastatic recurrence, receiver operating curve (ROC) analysis was used. As shown in Fig. 5, patients with post-CRT CD133, OCT4, and SOX2 above cut-off value ('High') showed significantly worse disease free survival, compared with those with 'Low'. No such relationship was observed for post-CRT CD44.

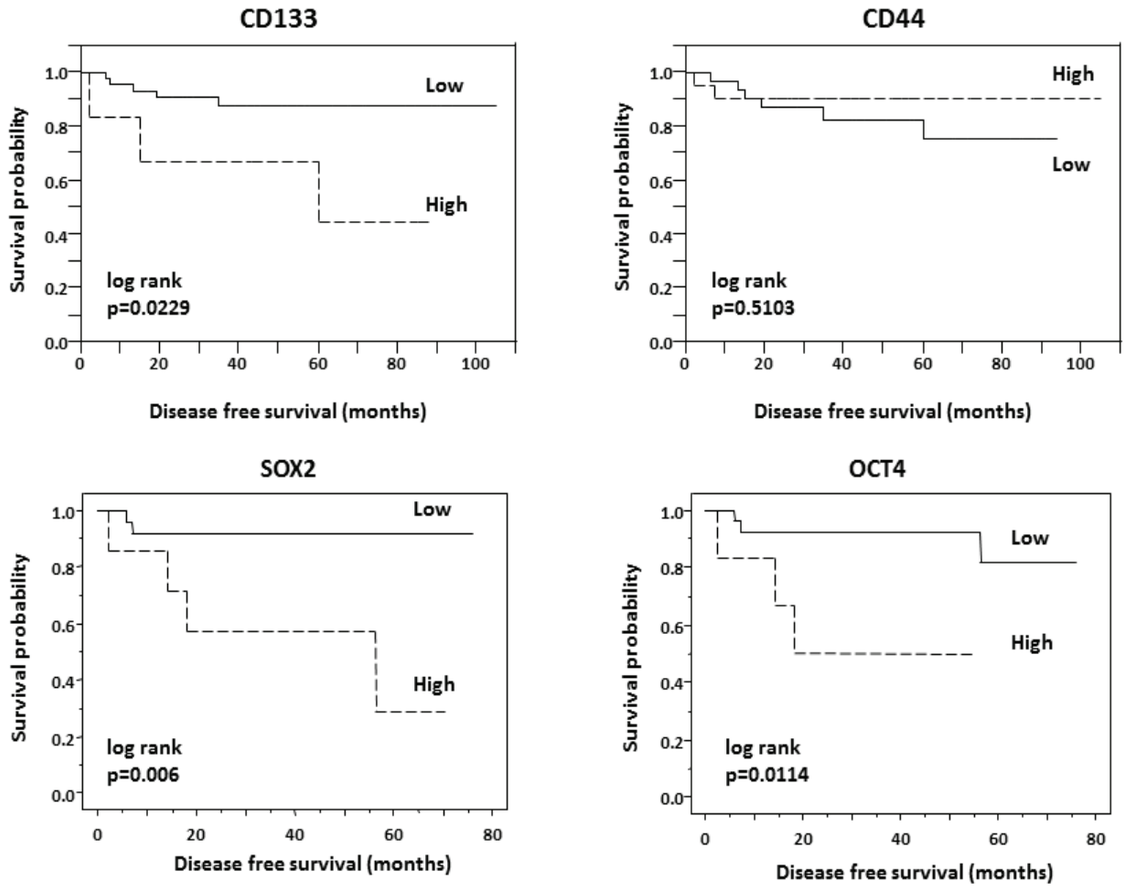

Fig. 5. Kaplan-Meier plots of disease free survival according to post-CRT CD133, CD44, SOX2, and OXT4 expression

Introduction of preoperative CRT followed by surgery (total mesorectal excision) in the management of rectal cancer significantly decreased local recurrence rate and improved patients' survival. However, the rate of distant metastatic recurrence still remains as high as 15-20% of rectal cancer treated with preoperative CRT followed by TME (Guillem et al., 2005). Identifying predictive markers for disease recurrence or poor prognosis of such patients is urgently required for appropriate treatment stratification.

Our results suggest that the expression of 'stem cell' genes such as CD133, OCT4 and SOX2 on post-CRT residual cancer cells may predict metachronous distant metastasis and poor prognosis of rectal cancer patients treated with preoperative CRT followed by surgery.

7. Clinical significance of residual cancer cells after CRT as putative colorectal CSCs

7.1 The proportion of putative CSCs in primary tumors

The presence of CSCs in primary tumors seems to be of prognostic significance for several malignancies (Liu et al., 2007; Zeppernick et al., 2008). In primary colorectal cancer, CD133 expression has also been reported to be a significant prognostic marker (Horst et al., 2008; Kojima et al., 2008). This may indicate that the proportion of CD133 expressing CSCs in primary, non-treatment tumor might be predictive for less treatment efficacy, more chance of disease recurrence, and poor prognosis of CRC patients.

In this study, the expression of CD133, CD44 SOX2, and OCT4 in pre-CRT primary tumor did not correlate with disease recurrence or survival of rectal cancer patients (data not shown). Since we had only 30 pre-CRT endoscopic tumor biopsies available, our data should be interpreted with caution. However, post-CRT, but not pre-CRT CD133, SOX2, and OCT4 has shown to be associated with metachronous distant metastasis and poor prognosis of rectal cancer patients treated with preoperative CRT followed by surgery.

7.2 The proportion of putative CSCs in post-treatment residual tumors

According to the CSC hypothesis, CRT surviving cancer cells (residual cancer cells following CRT) should contain a higher frequency of CRT-resistant colorectal CSCs, compared with primary, pre-CRT cancer cells. Our correlation results between pre-CRT or post-CRT CD133, SOX2, OCT4, VEGF, and EGFR, show that CRT may eliminate a majority of cancer cells expressing VEGF or EGFR (considered non-CSCs with CRT sensitive phenotype), and may leave a small population of cancer cells expressing CD133, OCT4, or SOX2 (considered CSCs with CRT resistant phenotype).

Although we think that residual cancer cells are not completely identical to CSCs, our results suggest that the relative proportion of putative CSCs expressing CD133, OCT4, or SOX2 may increase in post-CRT residual cancer cells in FFPE specimens, compared with pre-CRT primary tumor cells. Our findings are consistent with recent experiments (Dallas et al., 2009; Dylla et al., 2008).

7.3 CD133 and CD44 as potential markers for colorectal CSCs

Both CD133 and CD44 are of functional importance as potential cell surface markers for colorectal CSC. It still remains to be resolved if either CD133 or CD44 could be clinically important in CRT surviving cells, or if CRT can increase the expression of these markers. In pre- and post-CRT paired specimens, significant increase in tumoral CD133 and significant decrease in tumoral CD44 was observed. *In vitro*, CD133, but not CD44 was increased in radiation-resistant surviving colorectal cancer cell lines (HT29 cells).

CD133 seemed preferable to CD44 as the marker of colorectal CSCs according to the notion that CRT may increase the relative proportion of CSCs which express the potential markers of colorectal CSCs.

8. Conclusion

The proportion of CSCs in residual tumors following preoperative CRT may be more accurately predictive for less treatment efficacy, more chance of disease recurrence, and poor prognosis of rectal cancer patients than the proportion of CSCs in non-pretreatment

primary tumor. Expression of the potential markers of colorectal CSCs in microdissected residual cancer on FFPE specimens may provide useful information regarding treatment stratification and clinical management of rectal cancer patients after CRT and surgery.

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Cancer Stem Cells - The Cutting Edge

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Over the last thirty years, the foremost inspiration for research on metastasis, cancer recurrence, and increased resistance to chemo- and radiotherapy has been the notion of cancer stem cells.The twenty-eight chapters assembled in Cancer Stem Cells - The Cutting Edge summarize the work of cancer researchers and oncologists at leading universities and hospitals around the world on every aspect of cancer stem cells, from theory and models to specific applications (glioma), from laboratory research on signal pathways to clinical trials of bio-therapies using a host of devices, from solutions to laboratory problems to speculation on cancersâ€™ stem cellsâ€™ evolution. Cancer stem cells may or may not be a subset of slowly dividing cancer cells that both disseminate cancers and defy oncotoxic drugs and radiation directed at rapidly dividing bulk cancer cells, but research on cancer stem cells has paid dividends for cancer prevention, detection, targeted treatment, and improved prognosis.

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