SPANDIDOS PUBLICATIONS

ONCOLOGY LETTERS 14: 7791-7798, 2017

Leucine-rich repeat-containing G protein-coupled receptor 5 and CD133 expression is associated with tumor progression and resistance to preoperative chemoradiotherapy in low rectal cancer

YUZO HARADA[1], SHINSUKE KAZAMA[1], TEPPEI MORIKAWA[2], KOJI MURONO[1], KOJI YASUDA[1], KENSUKE OTANI[1], TAKESHI NISHIKAWA[1], TOSHIAKI TANAKA[1], TOMOMICHI KIYOMATSU[1], KAZUSHIGE KAWAI[1], KEISUKE HATA[1], HIROAKI NOZAWA[1], HIRONORI YAMAGUCHI[1], SOICHIRO ISHIHARA[1] and TOSHIAKI WATANABE[1]

[1]Division of Surgical Oncology, Department of Surgery, Faculty of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo 113-8656; [2]Department of Pathology, The University of Tokyo Hospital, Bunkyo-ku, Tokyo 113-8655, Japan

Received May 6, 2017; Accepted October 3, 2017

DOI: 10.3892/ol.2017.7207

Abstract. Preoperative chemoradiotherapy has been performed as a standard therapy for advanced low rectal cancer. Cancer stem cells (CSCs) have been reported to contribute to resistance to treatment and patient prognosis. Leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) and cluster of differentiation (CD133) are putative markers for CSCs. However, their prognostic ability remains unknown, and evaluation of a single marker can be insufficient due to the heterogeneity of cancer. LGR5 and CD133 expression was immunohistochemically evaluated in surgical specimens of 56 patients who received curative resection following chemoradiotherapy for advanced low rectal cancer. In addition, the correlations between their expression levels, and clinicopathological features and patient prognosis were asessed. LGR5 expression was significantly correlated with lymphatic invasion, lymph node metastasis, and tumor node metastasic (TNM) stage. CD133 expression was significantly correlated with vascular invasion and the tumor regression grade. Combined expression was significantly correlated with lymphatic invasion, tumor regression grade and TNM stage, but not with overall, and disease-free survival. LGR5 and CD133 expressions may represent useful markers associated with tumor progression and resistance to chemoradiotherapy in patients with low rectal cancer. Furthermore, combined expression of these markers may be a more useful marker compared with the expression of each single marker.

Correspondence to: Dr Yuzo Harada, Division of Surgical Oncology, Department of Surgery, Faculty of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan
E-mail: haraday-sur@h.u-tokyo.ac.jp

Key words: LGR5, CD133, rectal cancer, chemoradiotherapy, immunohistochemistry

Introduction

Recently, in the treatment of advanced low rectal cancer, preoperative chemoradiotherapy (CRT) has been widely accepted as a standard therapy. Previous studies have shown that preoperative CRT contributes to tumor down-staging and decreases locoregional recurrence post-surgery (1). Although the clinical significance of the CRT response has not been fully elucidated, several reports described that it may represent a predictor of clinical outcome, including tumor recurrence and patient survival. However, patients show different responses to CRT, with some cases showing little or no response (2). Therefore, prediction of the CRT response is warranted to avoid unnecessary treatment and adverse events such as radiation dermatitis, hematologic toxicity, and enteritis.

The cancer stem cell (CSC) theory, proposed by Hamburger *et al*, states that tumor cells are not only heterogeneous but also show a hierarchy, and CSCs, comprising a small part of tumors, are responsible for this heterogeneity due to their self-renewal and proliferation abilities (3). CSCs have been reported to be associated with tumor progression and recurrence. Although conventional cytotoxic therapies, such as chemotherapy or radiotherapy, target rapidly dividing cells, CSCs are estimated to be resistant to those therapies, as they divide more slowly (4,5). Finding a specific marker to identify CSCs is important, and prior studies have reported several putative CSC markers for colorectal cancer (CRC), including leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) and cluster of differentiation-133 (CD133) (6,7).

LGR5 is a glycoprotein hormone receptor with a seven transmembrane domain. Carmon *et al* reported that R-spondin proteins function as ligands of LGR5 and that LGR5 is a target of the Wnt/β-catenin signaling pathway, which is important for the maintenance of the colonic crypt (8). Barker *et al* demonstrated that LGR5 localized at the crypt base in the small intestine, and that LGR5-positive cells could generate multiple cell lineages of intestinal epithelium (9). Thus, LGR5 is considered a stem cell marker in the small intestine and colon. Of note, LGR5 is detected in many different tumors,

including CRC (10-13). In CRC, previous studies, including two meta-analyses, demonstrated that LGR5 expression was associated with tumor progression such as tumor depth, lymphovascular invasion, lymph node metastasis, advanced tumor stage, and poor overall survival (OS) (14-16). Moreover, prior studies have evaluated LGR5 expression in rectal cancer patients treated with preoperative CRT, and reported associations with worse sensitivity to CRT and poor patient prognosis (17).

CD133 is a five transmembrane glycoprotein that is widely detected in many tumors, including colon cancers (18-20). Prior studies have reported that CD133-positive colon cancer cells have self-renewal and proliferation abilities (7). In CRC, CD133 has also been reported to be associated with tumor depth, lymphovascular invasion, lymph node metastasis, and advanced tumor stage (21). Further, two meta-analyses demonstrated that CD133 expression correlated with worse OS (22,23). The correlations between CD133 expression and response to CRT have also been evaluated, with CD133 shown to associate with CRT resistance and poor patient prognosis (24-26).

However, these previous reports evaluated the clinical significance of LGR5 and CD133 expressions separately. To our knowledge, the significance of combined LGR5 and CD133expression remains unclarified. Therefore, herein, we evaluated both LGR5 and CD133 expressions immunohistochemically in low rectal cancer patients treated with preoperative CRT using serial sections, and analyzed the relationships between those expressions and clinicopathological features and patient prognosis.

Materials and methods

Patients and specimens. Sixty one consecutive patients underwent curative resection after CRT for advanced low rectal cancer in the Department of Surgical Oncology, University of Tokyo Hospital between March 2001 and October 2009. All patients were diagnosed as low rectal cancer, and the tumor depth was estimated to be deeper than the muscularis propria. Tumor depth, nodal status, and presence of distant metastases were determined by computed tomography and magnetic resonance imaging. They received a total radiation dose of 50.4 Gy (1.8 Gy in 28 fractions) and concomitant chemotherapy (oral administration of tegafur-uracil 300 mg/m2/day and leucovorin 75 mg/day). Total mesorectal excision with lymph node dissection was performed following an interval of 6-8 weeks post-CRT. All patients underwent regular follow-up examinations post-surgery. Tumor markers were examined every 3 months, and abdominal and chest computed tomography was performed every 6 months. Total colonoscopy was performed annually. Since we targeted the residual cancer tissue, 5 cases with no residual cancer cells after CRT were excluded. We analyzed 56 surgically resected specimens after CRT by immunohistochemistry.

All specimens were fixed in 10% formalin and embedded in paraffin. The histopathological findings were confirmed by the Department of Pathology, University of Tokyo. Data were collected from the patients' medical records. The TNM classification was determined according to the Union for International Cancer Control, 7th edition. The post-CRT histological tumor regression grade was evaluated according to the Japanese Classification of Colorectal Carcinoma, 8th edition (Grade 0: No necrosis or regressive change, 1a: >66.6% vital residual tumor cells, 1b: 33.3-66.6% vital residual tumor cells, 2: <33.3% vital residual tumor cells, 3: No vital residual tumor cells).

Table I. Characteristics of rectal cancer patients.

Characteristic	No. of patients (%)
Gender	
Male	37 (66.1)
Female	19 (33.9)
Mean age ± SD (years)	61.1±9.9
pT stage[a]	
T1-2	21 (37.5)
T3-4	35 (62.5)
Histological type	
Pap, Well	39 (69.6)
Mod, Por, Muc	17 (30.4)
Lymphatic invasion	
Present	5 (8.9)
Absent	51 (91.1)
Vascular invasion	
Present	31 (55.4)
Absent	25 (44.6)
Lymph node metastasis	
Present	9 (16.1)
Absent	47 (83.9)
Distant metastasis	
Present	6 (10.7)
Absent	50 (89.3)
TNM Stage[a]	
I-II	44 (78.6)
III-IV	12 (21.4)
Tumor regression grade[b]	
1a	17 (30.4)
1b	18 (32.1)
2	21 (37.5)

SD, standard deviation; Pap, Papillary adenocarcinoma; Well, Well differentiated adenocarcinoma; Mod, Moderately differentiated adenocarcinoma; Por, Poorly differentiated adenocarcinoma; Muc, Mucinous adenocarcinoma; TNM, tumor node metastasis; [a]According to TMN classification of malignant tumors, 7th edition according to UICC. [b]According to Japanese Classification of Colorectal Carcinoma, 8 th edition.

This study was approved by the ethics committee of the University of Tokyo on July 29, 2014 [No. 10476-(1)] and written informed consent was obtained from all patients.

LGR5 and CD133 Immunohistochemical staining. The tumor specimens were immunohistochemically stained, as described

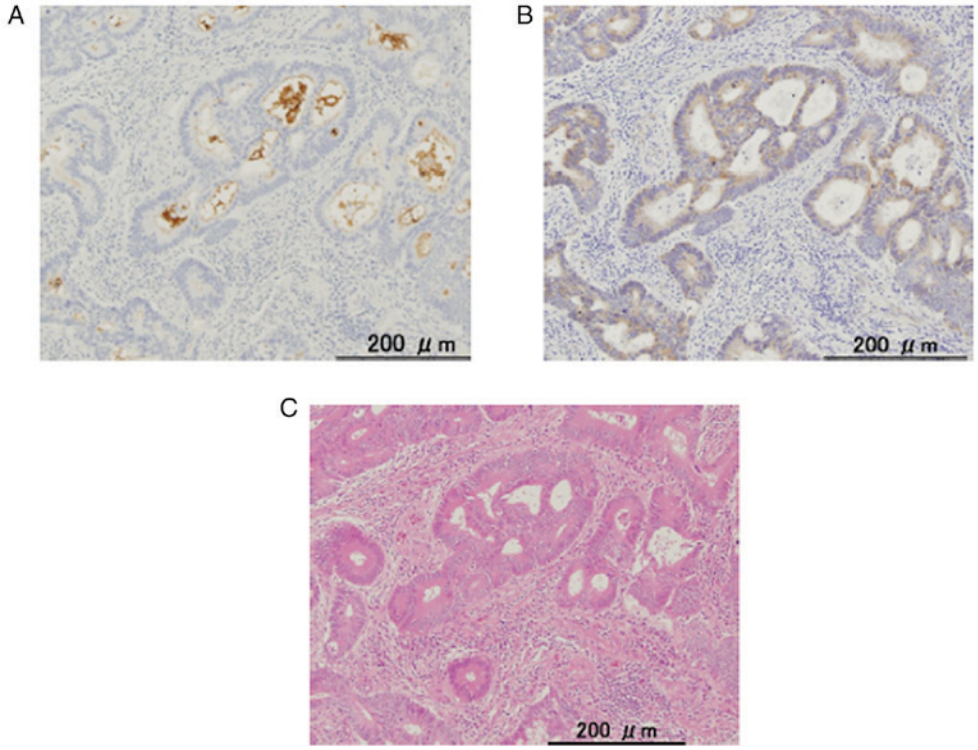

Figure 1. Immunohistochemical detection of leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) and cluster of differentiation-133 (CD133) in low rectal cancer. (A) Immunohistochemical staining of LGR5 (original magnification x100). LGR5 expression was detected in the cytoplasm. (B) Immunohistochemical staining of CD133 (original magnification x100). CD133 expression was detected on the luminal cell surface of the rectal tumor glands and on the intraglandular cellular debris. (C) Hematoxylin and eosin staining of a serial section of a primary lesion (original magnification x100).

below. The sections were deparaffinized in xylene, hydrated through a graded series of ethanol, and endogenous peroxidase was blocked. Heat-induced antigen retrieval was performed by incubation in sodium citrate buffer using an autoclave and non-specific proteins were blocked with 5% bovine serum albumin. Primary anti-LGR5 rabbit (Clone EPR3065Y, Epitomics, Burlingame, CA, USA) and anti-CD133 mouse monoclonal antibodies (Clone AC133, Miltenyi Biotec, Auburn, CA, USA) were added at dilutions of 1:100 and the sections were incubated overnight at 4°C. They were incubated with the Dako Envision kit (Dako, Carpinteria, CA, USA) following the manufacturer's recommendations. The reactivity was visualized in 2% 3,3'-diaminobenzidine tetrahydrochloride and 50 mM tris-buffer containing 0.3% hydrogen peroxidase. The crypt base of the normal colon mucosa and renal tubules were used as the positive controls for LGR5 and CD133, respectively. As a negative control, the antibody was replaced with PBS.

Evaluation of LGR5 and CD133 immunostaining. We defined positive expression of LGR5 as >50% positive cancer cells out of all cancer cells, since Saigusa *et al* also evaluated LGR5 expression in rectal cancer tissue after CRT using same cut-off value to clarify clinical significance of LGR5 (17). Positive expression of CD133 was defined as >5% positive cancer cells out of all cancer cells (27), since we previously showed CD133 expression in rectal cancer after CRT was associated with CRT response (22), and speculated the cut-off value to be appropriate. The sections were observed by a surgeon trained in pathology and a skillful pathologist, independently in a blinded fashion (S.K. and T.M.). Any discrepancies were resolved by discussion. Subsequently, we analyzed the correlations between the expressions of LGR5 and CD133 and clinicopathological factors and patient prognosis.

Statistical analysis. The correlations between LGR5 and CD133 expressions and clinicopathological factors were evaluated by the chi-squared test, Fisher's exact test, or unpaired t-test, as appropriate. OS and disease-free survival (DFS) were analyzed by the Kaplan-Meier method. P-values <0.05 were considered statistically significant. All analyses were performed using JMP 11.0 software (SAS Institute Inc., Cary, NC, USA).

Results

Clinicopathological findings. The clinicopathological factors are listed in Table I. The median patient age was 61 (range, 33-78) years, and 37 patients (66.1%) were male. After preoperative CRT, 21 (37.5%) and 35 (62.5%) patients had T1-2 and T3-4 tumors, respectively. Thirty-nine patients (69.6%) showed papillary carcinoma or well-differentiated adenocarcinoma histology. Nine (16.1%) and six (10.7%) patients had lymph node and distant metastases (3 liver, 1 lung, 1 brain, and 1 paraaortic lymph node metastases), respectively. Based on the response to CRT classification, 17 (30.4%), 18 (32.1%), and 21 (37.5%) patients were categorized as Grades 1a, 1b, and 2, respectively. The median follow-up period was 5.8 (range, 0.8-10.9) years.

Table II. LGR5 expression and clinicopathological features.

Characteristic	LGR5+ (n=36)		LGR5- (n=20)		P-value
	n	%	n	%	
Gender					
Male	25	69.4	12	60.0	0.48
Female	11	37.9	8	29.6	
Mean age ± SD (years)	60.2±11.1		62.8±7.1		0.51
pT stage					
T1-2	15	42.0	6	30.0	0.38
T3-4	21	58.0	14	70.0	
Histological type					
Pap, Well	24	66.7	15	75.0	0.51
Mod, Por, Muc	12	33.3	5	25.0	
Lymphatic invasion					
Present	5	13.9	0	0.0	0.03
Absent	31	86.1	20	100.0	
Vascular invasion					
Present	19	52.8	12	60.0	0.60
Absent	17	47.2	8	40.0	
Lymph node metastasis					
Present	9	25.0	0	0.0	<0.01
Absent	27	75.0	20	100.0	
Distant metastasis					
Present	5	13.9	1	5.0	0.28
Absent	31	86.1	19	95.0	
TNM Stage					
I-II	24	66.7	20	100	<0.01
III-IV	12	33.3	0	0	
Tumor regression grade[a]					
1a	11	30.6	6	30.0	0.94
1b	11	30.6	7	35.0	
2	14	38.9	7	35.0	

SD, standard deviation; Pap, Papillary adenocarcinoma; Well, Well differentiated adenocarcinoma; Mod, Moderately differentiated adenocarcinoma; Por, Poorly differentiated adenocarcinoma; Muc, Mucinous adenocarcinoma; LRG5, Leucine-rich repeat-containing G protein-coupled receptor 5, TNM, tumor node metastasis; [a]According to Japanese Classification of Colorectal Carcinoma, 8 th edition.

LGR5 and CD133 expressions. LGR5 expression was detected in the cytoplasm while CD133 expression was detected at the cell membrane and the intraglandular cellular debris in some tumor glands (Fig. 1). The staining pattern was mostly similar to that previously reported. As a result of the immunohistochemistry scoring, 36 (64.3%) and 29 (51.8%) patients were categorized as LGR5-positive and CD133-positive, respectively. Twenty-one patients (37.5%) showed positive expressions of both markers.

Relationships between LGR5 expression and clinicopathological features. LGR5 expression significantly correlated with lymphatic invasion (P=0.03), lymph node metastasis (P<0.01), and TNM stage (P<0.01) (Table II). No significant difference in OS or DFS was found [LGR5 positive vs. negative: 5-year OS 85.0% vs. 85.0% (P=0.86), 5-year DFS 63.9% vs. 70.0% (P=0.67), respectively].

Relationship between CD133 expression and clinicopathological features. CD133 expression significantly correlated with vascular invasion (P<0.01) and the tumor regression grade (P<0.01) (Table III). No significant difference in OS or DFS was found [CD133 positive vs. negative, 5-year OS 80.9% vs. 88.9% (P=0.40), 5-year DFS 58.6% vs. 73.9% (P=0.19), respectively].

Relationships between combined LGR5 and CD133 expression and clinicopathological features. No significant correlation was found between the expressions of the two markers (Table IV). The patients were divided into groups of positive

Table III. CD133 expression and clinicopathological features.

Characteristic	CD133+ (n=29)		CD133- (n=27)		P-value
	n	%	n	%	
Gender					
Male	18	62.1	19	70.4	0.51
Female	11	37.9	8	28.9	
Mean age ± SD (years)	61.9±10.2		60.3±9.6		0.48
pT stage					
T1-2	8	27.6	13	48.1	0.11
T3-4	21	72.4	14	51.9	
Histological type					
Pap, Well	19	65.5	20	74.1	0.49
Mod, Por, Muc	10	34.5	7	25.9	
Lymphatic invasion					
Present	4	13.8	1	3.7	0.17
Absent	25	86.2	26	96.3	
Vascular invasion					
Present	21	72.4	10	37.0	<0.01
Absent	8	27.6	17	63.0	
Lymph node metastasis					
Present	6	20.7	3	11.2	0.32
Absent	23	79.3	24	88.9	
Distant metastasis					
Present	4	13.8	2	7.4	0.44
Absent	25	86.2	25	92.6	
TNM Stage					
I-II	20	69	24	88.9	0.06
III-IV	9	31	3	11.1	
Tumor regression grade[a]					
1a	14	48.3	3	11.1	<0.01
1b	11	37.9	7	25.9	
2	4	13.8	17	63.0	

[a]According to Japanese Classification of Colorectal Carcinoma, 8 th edition. SD, standard deviation; Pap, Papillary adenocarcinoma, Well, Well differentiated adenocarcinoma; Mod, Moderately differentiated adenocarcinoma; Por, Poorly differentiated adenocarcinoma; Muc, Mucinous adenocarcinoma; CD, cluster of differentiation; TNM, tumor node metastasis.

expression of both markers and of all other patients, and the relationships between combined expression and the clinicopathological features were analyzed (Table V). Expression of both LGR5 and CD133 significantly correlated with lymphatic invasion (P=0.04), the tumor regression grade (P<0.01), and TNM stage (P<0.01). No significant difference in OS or DFS was found [LGR5 and CD133 positive vs. LGR5 or CD133 negative: 5-year OS 78.1% vs. 85.0% (P=0.53), 5-year DFS 57.1% vs. 71.3% (P=0.25), respectively].

Table IV. Correlation between LGR5 and CD133 expression.

LGR5 expression	CD133 expression +(%)	CD133 expression - (%)	P-value
+	21 (37.5)	15 (26.8)	0.19
-	8 (14.3)	12 (21.4)	

LRG5, Leucine-rich repeat-containing G protein-coupled receptor 5; CD, cluster of differentiation.

Discussion

Herein, we examined the expressions of LGR5 and CD133 in low rectal cancer patients treated with preoperative CRT immunohistochemically, using serial sections. LGR5 expression was associated with lymphatic invasion, lymph node metastasis, and TNM stage, while CD133 expression was

Table V. LGR5 and CD133 expression and clinicopathological features.

Characteristic	LGR5+ and CD133+ (n=21)		LGR- or CD133- (n=35)		P-value
	n	%	n	%	
Gender					
Male	14	66.7	23	65.7	0.94
Female	7	33.3	12	34.3	
Mean age ± SD (years)	61.4±11.0		61.0±9.2		0.82
pT stage					
T1-2	6	28.6	15	42.9	0.28
T3-4	15	71.4	20	57.1	
Histological type					
Pap, Well	12	57.1	27	77.1	0.12
Mod, Por, Muc	9	42.9	8	22.9	
Lymphatic invasion					
Present	4	19.0	1	2.9	0.04
Absent	17	81.0	34	97.1	
Vascular invasion					
Present	15	71.4	16	45.7	0.06
Absent	6	28.6	19	54.3	
Lymph node metastasis					
Present	6	28.6	3	8.6	0.05
Absent	15	71.4	32	91.4	
Distant metastasis					
Present	4	19.0	2	5.7	0.13
Absent	17	81.0	33	94.3	
TNM Stage					
I-II	12	57.1	32	91.4	<0.01
III-IV	9	42.9	3	8.6	
Tumor regression grade[a]					
1a	11	52.4	6	17.1	<0.01
1b	7	33.3	11	31.4	
2	3	14.3	18	51.4	

[a]According to Japanese Classification of Colorectal Carcinoma, 8 th edition. SD, standard deviation; Pap, Papillary adenocarcinoma; Well, Well differentiated adenocarcinoma; Mod, Moderately differentiated adenocarcinoma; Por, Poorly differentiated adenocarcinoma; Muc, Mucinous adenocarcinoma; TNM, tumor node metastasis; LRG5, Leucine-rich repeat-containing G protein-coupled receptor 5; CD, cluster of differentiation.

associated with vascular invasion. Although CD133 expression correlated with the response to CRT, LGR5 expression did not. Moreover, the co-expression of LGR5 and CD133 significantly correlated with lymphatic invasion, the tumor regression grade, and TNM stage. No significant correlation with OS or DFS was found.

We evaluated the expressions of LGR5 and CD133 in low rectal cancer using surgically resected specimens after CRT, since the assessment of very small biopsy samples obtained pre-CRT is difficult. There are several reports evaluating the expressions of these markers, separately, post-CRT. Saigusa *et al* (28) investigated LGR5 expression in rectal cancer patients treated with preoperative CRT and revealed a correlation with patient prognosis, while no correlation with any clinicopathological factor or prognosis was observed in CRC patients treated without preoperative therapy. They also described that CSCs were relatively increasing after CRT, because CSCs are resistant to CRT, as compared to non-CSCs (28). Kawamoto *et al* (25) evaluated CD133 expression in rectal cancer patients treated with preoperative CRT and described that, while CD133 expression in pre-CRT specimens before CRT did not associate with prognosis, those obtained post-CRT associated with DFS.

Regarding clinicopathological factors, prior studies in CRC patients have demonstrated correlations between LGR5 and CD133 expressions and tumor depth, lymph

node metastasis, lymphatic invasion, vascular invasion, and advanced tumor stage (14-16,21,23,24). However, the precise mechanisms of these correlations are still under investigation. Hirsch *et al* showed that silencing of LGR5 reduced proliferation, migration, and colony formation in CRC cell lines (29). The correlation between LGR5 and tumor proliferation in thyroid cancers has also been reported (12). Carmon *et al* (8) demonstrated that LGR5 enhanced cell proliferation through Wnt/β-catenin signaling (8), and LGR5 has been reported to promote epithelial-mesenchymal transition through activation of Wnt/β-catenin signaling in breast cancer (13). Moreover, Xi *et al* (10) showed a correlation between LGR5 expression and matrix metalloproteinase-2 in gastric cancer, and declared that LGR5 played an important role in tumor invasion and metastasis through matrix metalloproteinase-2, which degrades type IV collagen of the extracellular matrix and basal membrane. With respect to CD133, Chao *et al* (30) reported that CD133+ colon cancer cells had an enhanced ability to interact with neighboring fibroblasts, indicating that CD133+ cells are more invasive than CD133-cells. In lung and pancreatic cancers, CD133 expression has been shown to relate to vasculogenesis (27) and to be an important factor for epithelial-mesenchymal transition (31). Our findings were consistent with those of these previous studies.

Regarding the response to CRT, Saigusa *et al* reported that patients with high LGR5 expression had a poor pathological response to CRT (17). Hongo *et al* (24) reported that high CD133 expression correlated with worse response to CRT. Although the mechanisms remain unclear, there are several reports indicating resistance to chemotherapy and radiotherapy of CSCs. LGR5 expression has been reported to increase after radiation in CRC cell lines (28), and LGR5-positive CRC cells are reportedly resistant to chemotherapy through the ATP-binding cassette family (32). Moreover, Bao *et al* (5) demonstrated that CD133-positive glioma cells showed reduced sensitivity to radiation through activation of DNA damage repair. In CRC cell lines, CD133 expression has been reported to increase after irradiation (33), and CD133-positive CRC cells show reduced sensitivity to chemotherapy (34).

Herein, we could not show the correlation between LGR5 expression and the response to CRT, although CD133 expression was associated with a poor response. This can be attributed to several factors. First, the role of LGR5 in chemotherapy resistance is controversial, although Planutis *et al* (35) reported that LGR5-expressing CRC cells were more sensitive to anticancer drugs. Second, single irradiation *in vitro* is different from multiple courses of irradiation in clinical settings. Third, CRT regimens are not identical with respect to the concomitant chemotherapy and frequency of radiotherapy. Finally, our study had a relatively small sample size, which may have affected the statistical power. Regarding the patient prognosis, prior studies, including several meta-analyses, have demonstrated that these markers are poor prognostic factors (15,16,22,23). However, we could not show these correlations due to the small sample size.

Several putative cancer stem cell markers, including LGR5 and CD133, have been reported, but their clinical significance is not fully understood. Because of the heterogeneity of CRC, a single marker for CSCs may not be sufficient for precise evaluation. To our knowledge, there is no previous report evaluating both LGR5 and CD133 expression in specimens of CRC patients. In CRC cell lines, Kobayashi *et al* reported that LGR5+/CD133+ and LGR5-/CD133+ cells formed colonies, while almost all LGR5-/CD133-cells could not; their results moreover showed that the colony formation of LGR5+/CD133+ cells was higher than that of LGR5-/CD133+ cells (36). Therefore, we suppose that the subgroup with both LGR5 and CD133 expression are at high risk of malignancy. Accordingly, patients with both LGR5 and CD133 expression had significantly more lymphatic invasion, worse tumor regression grade and more advanced TNM stage, and tended to have more vascular invasion and lymph node metastasis. Although more correlations between the combined expression and clinicopathological factors were found than in the analysis of the single markers, further studies are necessary.

Our data should be interpreted with caution, as this study has several limitations. First, this was a retrospective study with a relatively small patient number. Second, we investigated protein expression using immunohistochemistry, but did not investigate the gene expression. Third, there are different methods of evaluating positive immunohistochemical staining of LGR5 and CD133. Therefore, prospective studies with a larger number of patients and the same methodology are needed.

We here demonstrated that the expression of LGR5 was associated with lymphatic invasion, lymph node metastasis, and TNM stage, while CD133 associated with the response to CRT in low rectal cancer. Moreover, combined expression of LGR5 and CD133 may be a more useful marker than the evaluation of a single marker.

References

1. Bosset JF, Collette L, Calais G, Mineur L, Maingon P, Radosevic-Jelic L, Daban A, Bardet E, Beny A and Ollier JC; EORTC Radiotherapy Group Trial 22921: Chemotherapy with preoperative radiotherapy in rectal cancer. N Engl J Med 355: 1114-1123, 2006.
2. Tomono A, Yamashita K, Kanemitsu K, Sumi Y, Yamamoto M, Kanaji S, Imanishi T, Nakamura T, Suzuki S, Tanaka K and Kakeji Y: Prognostic significance of pathological response to preoperative chemoradiotherapy in patients with locally advanced rectal cancer. Int J Clin Oncol 21: 344-349, 2016.
3. Hamburger AW and Salmon SE: Primary bioassay of human tumor stem cells. Science 197: 461-463, 1977.
4. Reya T, Morrison SJ, Clarke MF and Weissman IL: Stem cells, cancer, and cancer stem cells. Nature 414: 105-111, 2001.
5. Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, Dewhirst MW, Bigner DD and Rich JN: Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature 444: 756-760, 2006.
6. Espersen ML, Olsen J, Linnemann D, Høgdall E and Troelsen JT: Clinical implications of intestinal stem cell markers in colorectal cancer. Clin Colorectal Cancer 14: 63-71, 2015.
7. Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C and De Maria R: Identification and expansion of human colon-cancer-initiating cells. Nature 445: 111-115, 2007.
8. Carmon KS, Gong X, Lin Q, Thomas A and Liu Q: R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/beta-catenin signaling. Proc Natl Acad Sci USA 108: 11452-11457, 2011
9. Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, Haegebarth A, Korving J, Begthel H, Peters PJ and Clevers H: Identification of stem cell in small intestine and colon by marker gene Lgr5. Nature 449: 1003-1017, 2007.
10. Xi HQ, Cai AZ, Wu XS, Cui JX, Shen WS, Bian SB, Wang N, Li JY, Lu CR, Song Z, *et al*: Leucine-rich repeat-containing G-protein-coupled receptor 5 is associated with invasion, metastasis, and cloud be a potential therapeutic target in human gastric cancer. Br J Cancer 110: 2011-2020, 2014.

11. McClanahan T, Koseoglu S, Smith K, Grein J, Gustafson E, Black S, Kirschmeier P and Samatar AA: Identification of overexpression of orphan G protein-coupled receptor GPR49 in human colon and ovarian primary tumors. Cancer Biol Ther 5: 419-426, 2006.
12. Michelotti G, Jiang X, Sosa JA, Diehl AM and Henderson BB: LGR5 is associated with tumor aggressiveness in papillary thyroid cancer. Oncotarget 6: 34549-34560, 2015.
13. Yang L, Tang H, Kong Y, Xie X, Chen J, Song C, Liu X, Ye F, Li N, Wang N and Xie X: LGR5 promotes breast cancer progression and maintains stem-like cells through activation of Wnt/β-catenin signaling. Stem Cells 33: 2913-2924, 2015.
14. Wu XS, Xi HQ and Chen L: Lgr5 is a potential marker of colorectal carcinoma stem cells that correlates with patient survival. World J Surg Oncol 10: 244, 2012.
15. Chen Q, Zhang X, Li WM, Ji YQ, Cao HZ and Zheng P: Prognostic value of LGR5 in colorectal cancer: A meta-analysis. PLoS One 9: e107013, 2014.
16. Han Y, Xue X, Jiang M, Guo X, Li P, Liu F, Yuan B, Shen Y, Guo X, Zhi Q and Zhao H: LGR5, a relevant marker of cancer stem cells, indicates a poor prognosis in colorectal cancer patients: A meta-analysis. Clin Res Hepatol Gastroenterol 39: 267-273, 2015.
17. Saigusa S, Inoue Y, Tanaka K, Toiyama Y, Matsushita K, Kawamura M, Okugawa Y, Hiro J, Uchida K, Mohri Y and Kusunoki M: Clinical significance of LGR5 and CD44 expression in locally advanced rectal cancer after preoperative chemoradiotherapy. Int J Oncol 41: 1643-1652, 2012.
18. Ma S: Biology and clinical implications of CD133(+) liver cancer stem cells. Exp Cell Res 319: 126-132, 2013.
19. Long H, Xie R, Xiang T, Zhao Z, Lin S, Liang Z, Chen Z and Zhu B: Autocrine CCL5 signaling promotes invasion and migration of CD133+ ovarian cancer stem-like cells via NF-κB-mediated MMP-9 upregulation. Stem Cells 30: 2309-2319, 2012.
20. Shien K, Toyooka S, Ichimura K, Soh J, Furukawa M, Maki Y, Muraoka T, Tanaka N, Ueno T, Asano H, *et al*: Prognostic impact of cancer stem cell-related markers in non-small cell lung cancer patients treated with induction chemoradiotherapy. Lung Cancer 77: 162-167, 2012.
21. Zhou F, Mu YD, Liang J, Liu ZX, Chen HS and Zhang JF: Expression and prognostic value of tumor stem cell markers ALDH1 and CD133 in colorectal carcinoma. Oncol Lett 7: 507-512, 2014.
22. Chen S, Song X, Chen Z, Li X, Li M, Liu H and Li J: CD133 expression and the prognosis of colorectal cancer: A systematic review and meta-analysis. PLoS One 8: e56380, 2013.
23. Wang K, Xu J, Zhang J and Huang J: Prognostic role of CD133 expression in colorectal cancer: A meta-analysis. BMC Cancer 12: 573, 2012.
24. Hongo K, Kazama S, Sunami E, Tsuno NH, Takahashi K, Nagawa H and Kitayama J: Immunohistochemical detection of CD133 is associated with tumor regression grade after chemoradiotherapy in rectal cancer. Med Oncol 29: 2849-2857, 2012.
25. Kawamoto A, Tanaka K, Saigusa S, Toiyama Y, Morimoto Y, Fujikawa H, Iwata T, Matsushita K, Yokoe T, Yasuda H, *et al*: Clinical significance of radiation-induced CD133 expression in residual rectal cancer cells after chemoradiotherapy. Exp Ther Med 3: 403-409, 2012.
26. Saigusa S, Tanaka K, Toiyama Y, Yokoe T, Okugawa Y, Koike Y, Fujikawa H, Inoue Y, Miki C and Kusunoki M: Clinical significance of CD133 and hypoxia inducible factor-1α gene expression in rectal cancer after preoperative chemoradiotherapy. Clin Oncol (R Coll Radiol) 23: 323-332, 2011.
27. Maeda S, Shinchi H, Kurahara H, Mataki Y, Maemura K, Sato M, Natsugoe S, Aikou T and Takao S: CD133 expression is correlated with lymph node metastasis and vascular endothelial growth factor-C expression in pancreatic cancer. Br J Cancer 98: 1389-1397, 2008.
28. Saigusa S, Inoue Y, Tanaka K, Toiyama Y, Kawamura M, Okugawa Y, Okigami M, Hiro J, Uchida K, Mohri Y and Kusunoki M: Significant correlation between LKB1 and LGR5 gene expression and the association with poor recurrence-free survival in rectal cancer after preoperative chemoradiotherapy. J Cancer Res Clin Oncol 139: 131-138, 2013.
29. Hirsch D, Barker N, McNeil N, Hu Y, Camps J, McKinnon K, Clevers H, Ried T and Gaiser T: LGR5 positivity defines stem-like cells in colorectal cancer. Carcinogenesis 35: 849-858, 2014.
30. Chao C, Carmical JR, Ives KL, Wood TG, Aronson JF, Gomez GA, Djukom CD and Hellmich MR: CD133+ colon cancer cells are more interactive with the tumor microenvironment than CD133-cells. Lab Invest 92: 420-436, 2012.
31. Liu YM, Li XF, Liu H and Wu XL: Ultrasound-targeted microbubble destruction-mediated downregulation of CD133 inhibits epithelial-mesenchymal transition, stemness and migratory ability of liver cancer stem cells. Oncol Rep 34: 2977-2986, 2015.
32. Liu YS, Hsu HC, Tseng KC, Chen HC and Chen SJ: Lgr5 promotes cancer stemness and confers chemoresistance through ABCB1 in colorectal cancer. Biomed Pharmacother 67: 791-799, 2013.
33. Saigusa S, Tanaka K, Toiyama Y, Yokoe T, Okugawa Y, Kawamoto A, Yasuda H, Morimoto Y, Fujikawa H, Inoue Y, *et al*: Immunohistochemical features of CD133 expression: Association with resistance to chemoradiotherapy in rectal cancer. Oncol Rep 24: 345-350, 2010.
34. Lim SH, Jang J, Park JO, Kim KM, Kim ST, Park YS, Lee J and Kim HC: CD133-positive tumor cell content is a predictor of early recurrence in colorectal cancer. J Gastrointest Oncol 5: 447-456, 2014.
35. Planutis AK, Holcombe RF, Planoutene MV and Planoutis KS: SW480 colorectal cancer cells that naturally express Lgr5 are more sensitive to the most common chemotherapeutic agents than Lgr5-negative SW480 cells. Anticancer Drugs 26: 942-947, 2015.
36. Kobayashi S, Yamada-Okabe H, Suzuki M, Natori O, Kato A, Matsubara K, Jau Chen Y, Yamazaki M, Funahashi S, Yoshida K, *et al*: LGR5-positive colon cancer stem cells interconvert with drug-resistant LGR5-negative cells and are capable of tumor reconstitution. Stem Cells 30: 2631-2644, 2012.