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ORIGINAL ARTICLE

LGR5 and CD133 as prognostic and predictive markers for fluoropyrimidine-based adjuvant chemotherapy in colorectal cancer

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ABSTRACT

Background: Expression of leucine-rich-repeat-containing G-protein-coupled receptor 5 (LGR5) gene is associated with a metastatic phenotype and poor prognosis in colorectal cancer (CRC). CD133 expression is a putative cancer stem cell marker and a proposed prognostic marker in CRC, whereas the predictive value of CD133 expression for effect of adjuvant chemotherapy in CRC is unclear.

Material and methods: For the study of LGR5 mRNA and CD133 expression, tissue microarrays from 409 primary CRC stage II and III tumors, where patients had been randomized to adjuvant chemotherapy or surgery only, were available. LGR5 mRNA and CD133 expression were assessed by in situ hybridization (ISH) and immunohistochemistry (IHC), respectively. LGR5 mRNA and CD133 expression as prognostic and predictive markers were evaluated by univariate and multivariate analyses.

Results: For all CRC patients, positive LGR5 mRNA and CD133 expression were associated with classic adenocarcinoma histology type ($p = 0.001$ and $p = 0.014$, respectively). Positive LGR5 mRNA expression was also associated with smaller tumor diameter for CRC stage II ($p = 0.005$), but not for CRC stage III ($p = 0.054$). For CRC stage II, lack of LGR5 mRNA expression was associated with longer time to recurrence (TTR) in Kaplan-Meier ($p = 0.045$) and in multivariate Cox analysis (HR 0.27, 95% CI 0.08–0.95, $p = 0.041$). For colon cancer stage III patients, lack of CD133 expression was associated with better effect of adjuvant chemotherapy ($p = 0.016$) in Kaplan-Meier univariate analysis, but the interaction between CD133 and adjuvant chemotherapy was not statistically significant in multivariate analysis (HR 0.59, 95% CI 0.18–1.89, $p = 0.374$).

Conclusion: LGR5 mRNA expression is a prognostic factor for CRC stage II patients, whereas the value of CD133 expression as prognostic and predictive biomarker is inconclusive.

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Colorectal cancer (CRC) is a common malignancy with a high health burden. CRC is primarily treated by surgery whereas systemic adjuvant chemotherapy is administered for colon cancer stage III and high-risk colon cancer stage II patients [1–3]. However, many colon cancer stage III patients do not benefit from adjuvant chemotherapy as they already are cured by surgery or develop metastases despite the given chemotherapy. For colon cancer high-risk stage II patients, the gain of adjuvant chemotherapy is still uncertain [4]. Neoadjuvant chemotherapy for colon cancer is currently under investigation [5,6]. For rectal cancer, preoperative radiation alone or combined with chemotherapy are used, while adjuvant postoperative chemotherapy is still controversial [7]. Therefore, prognostic and preferably predictive factors are warranted in both CRC stage II and III.

Recently, a consensus of CRC molecular subtypes has been published [8]. The subtype with the highest degree of expression of mesenchymal and stem cell genes has the worst prognosis [8]. In CRC, cancer stem cells (CSCs) arise from dedifferentiated progenitor cells [9,10]. A subfraction of CSCs, long-term tumor initiating cells (LT-TIC) drive migration and metastases formation [11].

Leucine-rich-repeat-containing G-protein-coupled receptor 5 (LGR5) is a receptor for R-spondin and modulates downstream WNT signaling [12,13]. LGR5 positive cells are the fast-cycling columnar-based stem cells of colon mucosa; the population that is instrumental in neoplasia initiation [13,14]. High expression of stem cell genes (including LGR5) in CRC is associated with a metastatic phenotype and poor prognosis [15]. However, a clinical study utilizing in situ hybridization (ISH) for LGR5 mRNA expression did not confirm the prognostic value of LGR5 expression in CRC [16].

CD133 (prominin-1) was first classified as a marker for hematopoietic and neuronal stem cells [17]. It has also been recognized as a putative marker for colonic CSCs or tumor initiating cells [17,18]. The tumor promoting effects are proposed to be exerted, at least partly, through stabilization of β-catenin [19]. High CD133 expression is associated with high rate of metastases and disease progression in CRC [20,21]. Lack of CD133 expression predicted response to chemotherapy in a CRC stage III cohort, but the study was not randomized [22].

Our series of patients were randomized to only surgical resection of primary CRC or to treatment with fluorouracil

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(5-FU) and levamisole (Lev) after primary surgery. The aim of the present study was to evaluate the impact of LGR5 mRNA and CD133 expression on survival and response to 5-FU based treatment in CRC.

Material and methods

Study population

A prospective, randomized multicenter trial recruited 412 CRC patients from December 1992 to October 1996 in Norway, as previously described [23]. In brief, the patients had radical resections for adenocarcinoma of colon or rectum, verified by pathological examination. Synchronous metastases were excluded by chest x-ray, ultrasound or computed tomography (CT) of the abdomen, the CEA blood test and perioperative colonoscopy. Exclusion criteria were age less than 18 or more than 75 years. All patients were eligible for systemic chemotherapy. The study was approved by the Regional Ethics Committee, the Norwegian Medicines Control Authority and the Data Inspectorate. All patients gave their written and informed consent.

Chemotherapy and follow-up

The Moertel regimen was strictly followed with administration, as described in previous publications [23–25]. The treatment started within 42 days from the date of surgery. The first course of 5-FU was administered intravenously (i.v.) within 5 minutes at a dose of 450 mg/m^2 for five consecutive days. Lev was administered orally at a dose of 50 mg three times daily within the first three days during the loading course. Maintenance therapy was administered from Day 28 and lasted for 48 weeks. It consisted of a weekly dose of 450 mg/m^2 5-FU and of three daily doses of 50 mg Lev for three days, every other week. The patients had clinical encounter with a physician every six months for the first three years, and every year until the fifth year of follow-up. A regular checkup consisted of clinical examination, CEA testing, chest x-ray, and ultrasound or CT of abdomen. Patients had colonoscopy three years from the date of surgery.

Tissue microarray (TMA)

Tumor tissue samples of 409 CRC (99%) patients included in the randomized study were eligible for TMA construction. Up to three 1.0 mm tumor tissue cores per patient were taken from formalin-fixed, paraffin-embedded FFPE primary tumor samples and inserted in a recipient paraffin blocks using a standard precision instrument (Manual Tissue Arrayer MTA-1, Beecher Instruments, Inc., Sun Prairie, WI) [25,26]. Also, one tissue core from the same tumor tissue blocks, but of adjacent, macroscopically normal mucosa was inserted to the recipient paraffin blocks.

LGR5 mRNA in situ hybridization (ISH)

LGR5 mRNA expression was assessed by RNAscope 2.0 HD Detection Kit-Brown [Advanced Cell Diagnostics (ACD), Hayward, CA] according to the manufacturer's instructions. Briefly, the TMAs were deparaffinized and pretreated according to the manufacturer's protocol. The LGR5 probes were hybridized at 40 °C for 2 hours in a hybridization oven. After amplification, 3, 3′-diaminobenzidine (DAB) was added for the visualization of the target mRNA. Freshly cut sections of liver and colon cancer FFPE specimens were used as controls. Moreover, the probe targeting bacterial DapB (ACD) and the probe targeting human POLR2A (ACD) were used as negative and positive control, respectively. The specimens which exhibited negative staining with a negative control probe (DapB) and grade 1+ or higher staining with the positive control (POLR2A) probe, were considered adequate for LGR5 analysis. Two independent observers blinded to clinical endpoints evaluated the TMA slides. LGR5 mRNA expression was categorized into five grades: 0, 1+, 2+, 3+ and 4+, according to the manufacturer's guidelines. In subsequent analyses, LGR5 mRNA expression was categorized into negative expression (grade 0) and positive expression (1+–4+).

Immunohistochemistry (IHC)

TMA slides were deparaffinized and rehydrated. Antigen retrieval was performed using TE-buffer pH 9.0 (10 mM Tris, and 1 mM EDTA) [(pH 9.0), Dako, Glostrup, Denmark] in a kitchen pressure cooker. Subsequently, slides were incubated overnight at 4 °C with mouse anti-CD133 [(clone AC133), Miltenyi Biotec, Bergisch Gladbach, Germany] at a dilution 1:10, rinsed in TBST (0.05 M Tris-HCl, 0.15 M NaCl, 0.05% Tween 20, pH 7.5) and blocked with 3% H_2O_2 in distilled water for 10 minutes. Thereafter, slides were rinsed in TBST followed by 30 minutes incubation with anti-mouse polymeric horseradish peroxidase [(EnVision + System), Dako, Glostrup, Denmark] at room temperature. Immune complexes were visualized with DAB. All slides were counterstained with hematoxylin. Omission of the primary antibodies served as the negative control. The TMA slides were evaluated by two independent observers blinded to clinical endpoints. CD133 expression was characterized as positive when it exhibited staining on apical/endoluminal membrane of CRC with debris in ductal structure [27].

Statistical and survival analyses

We used Student's t-test and Pearson's exact χ^2-test to assess relations between the two biomarkers and the clinical variables. Pearson's product-moment correlation coefficient was used as a measure of an association between LGR5 mRNA and CD133. We included Ki-67 protein expression from our previous study into the analyses [26]. Pearson's exact χ^2-test and Student's t-test were utilized to test for goodness of fit of the samples successfully stained for LGR5 mRNA and CD133, where variables tested were: age, sex, TNM stage, tumor stage, tumor diameter, histology type, tumor differentiation, total number of lymph nodes, lymph node ratio (defined as the fraction of lymph nodes with metastasis divided by the total lymph node yield), Ki-67 staining and treatment. In two-sided tests, $p < 0.05$ was considered statistically significant.

Time to recurrence (TTR), calculated from the date of randomization to the date of the first loco-regional or distant recurrence, was used as the end point in survival analyses [28]. Patients dying of other causes before cancer recurrence were censored. Survival curves were compared using Kaplan-Meier plots and log-rank test. The predictive value of CD133 expression was evaluated by comparing five-year TTR of the arm with surgery only and the arm with postoperatively 5-FU/LEV treatment for the groups with negative and positive CD133 expression. Treatment effects were further estimated with Cox proportional hazards models. The SPSS 22 was used for statistical analysis (IBM Corp., Armonk, NY).

Results

LGR5 mRNA and CD133 staining in CRC

LGR5 mRNA was weakly expressed at the bottom of intestinal crypts in adjacent normal mucosa (Figure 1(a)). In CRC, 257 (63%) patient samples were successfully stained for LGR5 mRNA expression. The missing samples were missing at random, according to the goodness of fit tests (Data not shown). In tumor tissue, positive LGR5 mRNA expression was heterogeneous, from grade 1 + to 3 + per core (Figure 1(b)).

We did not observe any CD133 staining of epithelial or stromal cells in adjacent normal mucosa (Figure 1(c)). Three hundred and eighty-three samples (93%) were successfully stained for CD133 expression in CRC. The goodness of fit tests revealed that the missing samples were missing at random for all variables except total number of lymph nodes ($p = 0.008$). In tumor tissue, CD133 staining was positive on apical/endoluminal membrane in malignant ductal structures with debris in ducts (Figure 1(d)).

There was a weak association between CD133 protein expression and LGR5 mRNA expression [r (correlation coefficient) = 0.149, $p = 0.018$].

Associations between LGR5 mRNA, CD133 and clinical variables

For all CRC patients, positive LGR5 expression was associated with higher rate of classic adenocarcinoma histology type ($p = 0.001$, Table 1). For CD 133 expression, positive CD133 expression was associated with classic adenocarcinoma ($p = 0.014$), high/moderate tumor differentiation ($p = 0.017$) and lower percentage of Ki-67 staining ($p = 0.032$).

For colon cancer patients, positive LGR5 mRNA expression was associated with TNM stage III ($p = 0.024$, Table 2), smaller tumor diameter ($p = 0.016$) and higher rate classic adenocarcinoma histology type ($p < 0.001$). For CD133 expression, positive CD133 expression was associated with classic adenocarcinoma ($p = 0.046$), high/moderate tumor differentiation ($p = 0.014$) and lower percentage of Ki-67 staining ($p = 0.025$).

Furthermore, we investigated the relation between LGR5 mRNA expression and tumor diameter (Figure 2). There was especially strong association between positive LGR5 mRNA expression and statistically significant smaller tumor diameter for TNM stage II, both when CRC stage II and colon cancer

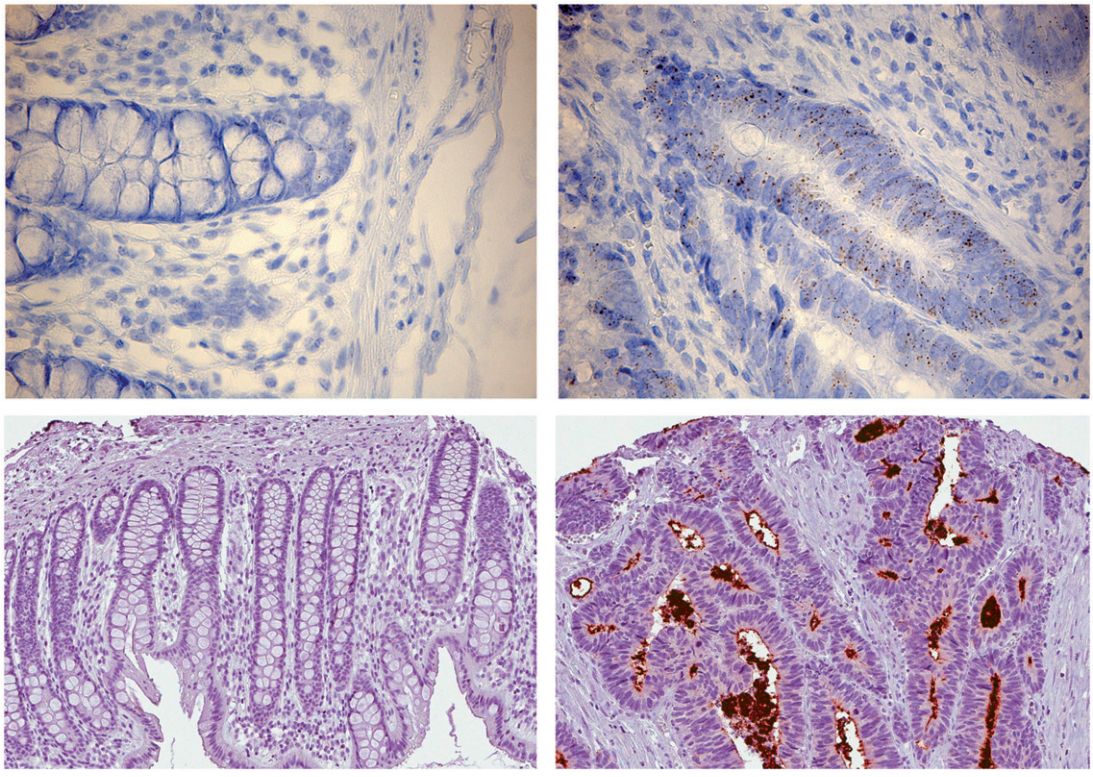

Figure 1. Images of representative LGR5 and CD133 staining (a) in situ hybridization (ISH) of LGR5 in normal adjacent mucosa (b) ISH of LGR5 in tumor tissue (c) immunohistochemistry (IHC) of CD133 in normal adjacent mucosa (d) IHC of CD133 in tumor tissue.

Table 1. LGR5 mRNA and CD133 expression in colorectal cancer according to clinical variables.

	LGR5			CD133		
Variable	Negative n= 66 N (%)	Positive n = 191 N (%)	p-Value[1,2]	Negative n = 224 N (%)	Positive n = 159 N (%)	p-Value[1,2]
Bowel segment			0.561			0.909
Colon	46 (74%)	136 (71%)		159 (71%)	112 (70%)	
Rectum	16 (26%)	55 (29%)		65 (29%)	47 (30%)	
Age[3]	59.7 (10.5)	61.3 (9.2)	0.268	61.2 (9.1)	61.4 (9.5)	0.760
Sex (female)	31 (50%)	87 (46%)	0.542	102 (46%)	81 (51%)	0.296
TNM stage			0.268			0.942
II	40 (65%)	108 (57%)		133 (59%)	95 (60%)	
III	22 (36%)	83 (43%)		91 (41%)	64 (40%)	
Tumor stage			0.853			0.301
T1 and T2	5 (8%)	17 (9%)		18 (8%)	13 (8%)	
T3	53 (86%)	165 (86%)		189 (84%)	140 (88%)	
T4	4 (7%)	9 (5%)		17 (8%)	6 (4%)	
Tumor diameter (cm)	9.2 (8.5)	6.9 (5.2)	0.061	7.7 (6.5)	7.5 (6.0)	0.767
Histology type			<0.001			0.014
Adenocarcinoma	48 (77%)	180 (94%)		196 (88%)	151 (95%)	
Variant[4]	14 (23%)	11 (6%)		28 (12%)	8 (5%)	
Tumor differentiation[5]			0.371			0.015
High/moderate	50 (83%)	166 (88%)		176 (80%)	142 (89%)	
Low	10 (17%)	23 (12%)		44 (20%)	17 (11%)	
Total number of lymph nodes[3]	9.5 (7.3)	9.2 (6.7)	0.717	9.1 (6.8)	8.7 (6.2)	0.773
Lymph node ratio[3,6]	16.1 (25.8)	14.8 (24.3)	0.730	18.3 (29.5)	13.6 (23.3)	0.439
Ki-67 staining			0.827			0.032
<40%	23 (43%)	70 (44%)		87 (44%)	74 (57%)	
≥40%	31 (57%)	88 (56%)		109 (56%)	57 (43%)	
Treatment (adjuvant therapy)	30 (48%)	97 (51%)	0.743	107 (48%)	85 (54%)	0.272

[1]Student's t-test;
[2]Pearson's exact χ^2-test;
[3]Mean, (SD);
[4]Variant includes signet-ring and mucinous carcinoma;
[5]Tumor differentiation has 4 missing values;
[6]Lymph node ratio is defined as the fraction of metastatic lymph nodes divided by the total lymph node yield.

Table 2. LGR5 mRNA and CD133 expression in colon cancer according to clinical variables.

	LGR5			CD133		
Variable	Negative n = 46 N (%)	Positive n = 136 N (%)	p-Value[1,2]	Negative n = 159 N (%)	Positive n = 112 N (%)	p-Value[1,2]
Age[3]	59.0 (10.8)	61.8 (9.4)	0.095	61.8 (9.2)	61.8 (9.3)	0.991
Sex (Female)	25 (54%)	65 (48%)	0.442	80 (50%)	62 (55%)	0.413
TNM stage			0.024			0.933
II	35 (76%)	78 (57%)		100 (63%)	71 (63%)	
III	11 (24%)	58 (43%)		59 (37%)	41 (37%)	
Tumor stage			0.924			0.462
T1 and T2	3 (6%)	7 (5%)		8 (5%)	5 (4%)	
T3	40 (88%)	121 (89%)		138 (87%)	102 (92%)	
T4	3 (6%)	8 (6%)		13 (8%)	5 (4%)	
Tumor diameter (cm)	11.9 (8.7)	8.2 (5.5)	0.016	9.6 (6.8)	9.3 (6.5)	0.940
Histology type[4]			<0.001			0.046
Adenocarcinoma	32 (70%)	126 (93%)		135 (85%)	104 (93%)	
Variant[5]	14 (30%)	10 (7%)		24 (15%)	8 (7%)	
Tumor differentiation[6]			0.183			0.014
High/moderate	34 (77%)	115 (86%)		117 (76%)	98 (88%)	
Low	10 (23%)	19 (14%)		38 (24%)	14 (12%)	
Total number of lymph nodes[3]	10.4 (8.0)	9.6 (6.9)	0.526	9.6 (6.9)	9.2 (6.5)	0.689
Ki-67 staining			0.857			0.025
<40%	18 (44%)	51 (46%)		62 (45%)	56 (60%)	
≥40%	23 (56%)	61 (54%)		77 (55%)	38 (40%)	
Lymph node ratio[3]	11.9 (24.6)	13.8 (23.5)	0.632	16.2 (28.1)	11.6 (21.9)	0.545
Treatment (adjuvant therapy)	20 (44%)	72 (53%)	0.267	75 (47%)	62 (55%)	0.184

[1]Student's t-test;
[2]Pearson's exact χ^2-test;
[3]Mean, (SD);
[4]Variant includes signet-ring and mucinous carcinoma;
[5]Tumor differentiation includes 4 missing values.

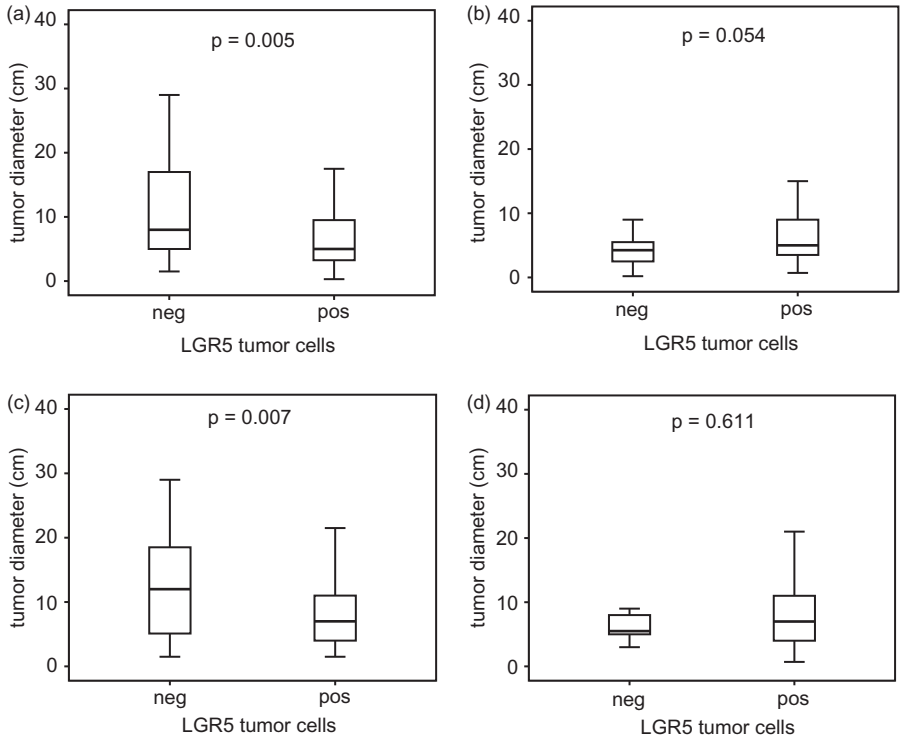

Figure 2. Correlation between LGR5 expression in tumor cells and tumor diameter (a) LGR5 and tumor diameter in CRC stage II (b) LGR5 and tumor diameter in CRC stage III (c) LGR5 and tumor diameter in colon stage II (d) LGR5 and tumor diameter in colon stage III.

stage II were investigated separately (p = 0.005 and p = 0.007, respectively).

LGR5 mRNA expression and influence on time to recurrence (TTR)

For CRC stage II patients, Kaplan-Meier analysis showed statistically significant better five-year TTR for the patients with lack of LGR5 mRNA expression compared to positive LGR5 expression (TTR 93% vs. 78%, p = 0.045, Figure 3). In the multivariate analysis, when adjusted for age, sex, treatment and Ki-67 expression, patients with lack of LGR5 mRNA expression had lower risk of recurrence than patients with positive LGR5 mRNA expression (HR 0.27, 95% CI 0.08–0.95, p = 0.041, Table 3).

For colon cancer stage II and rectal cancer stage II patients analyzed separately, no statistically significant differences were detected in five-year TTR for negative versus positive LGR5 mRNA expression [91% vs. 78%, p = 0.097 and 100% vs. 77%, p = 0.250 (Supplementary Figure 1, available online at http://www.informahealthcare.com), respectively]. LGR5 mRNA expression did not have any prognostic value for five-year TTR in either CRC stage III, in colon cancer stage III or rectal cancer stage III (p = 0.798, 0.770 and 0.620, respectively).

CD133 expression and influence on TTR

In predictive Kaplan-Meier analysis of colon cancer stage III patients, negative CD133 expression was associated with better effect of adjuvant therapy (p = 0.016, Figure 4). However, in multivariate analysis, when adjusted for clinical factors, no interaction between CD133 expression and treatment was demonstrated (HR 0.59, 95% CI 0.18–1.89, p = 0.374, Table 3).

For all CRC patients, CD133 expression was not a prognostic factor for five-year TTR (p = 0.875, Figure 4(c)). For colon cancer stage III patients regardless of treatment, there was no statistically significant difference in five-year TTR between the negative and positive CD133 expression (42% vs. 56%, p = 0.064, Figure 4(d)). In multivariate analysis, when adjusted for clinical factors regarded to be of importance, colon cancer stage III patients with negative CD133 expression had higher risk for relapse (HR 2.18, 95% CI 1.10–4.33, p = 0.026, Table 3). Further, for colon cancer stage III patients treated with surgery only, there was a difference in five-year TTR, although not statistically significant, between patients with negative versus positive CD133 expression (negative vs. positive, 32% vs. 50%, p = 0.051, Figure 4(e)). For colon cancer stage III patients which had adjuvant chemotherapy, five-year TTR did not differ between patients with negative versus positive CD133 expression (p = 0.595).

For multivariate analyses of all investigated cohorts see Supplementary Tables 1–6 (available online at http://www.informahealthcare.com).

Discussion

LGR5 and CD133 are putative markers for CSCs in CRC. In this study, we investigated prognostic and predictive value of these markers in a CRC cohort, randomized between adjuvant 5-FU/Lev therapy and surgery only. For CRC stage II, positive LGR5 mRNA expression was associated with smaller tumor diameter, while it was associated with worse five-year TTR. In Kaplan-Meier analysis for colon cancer stage III, we identified

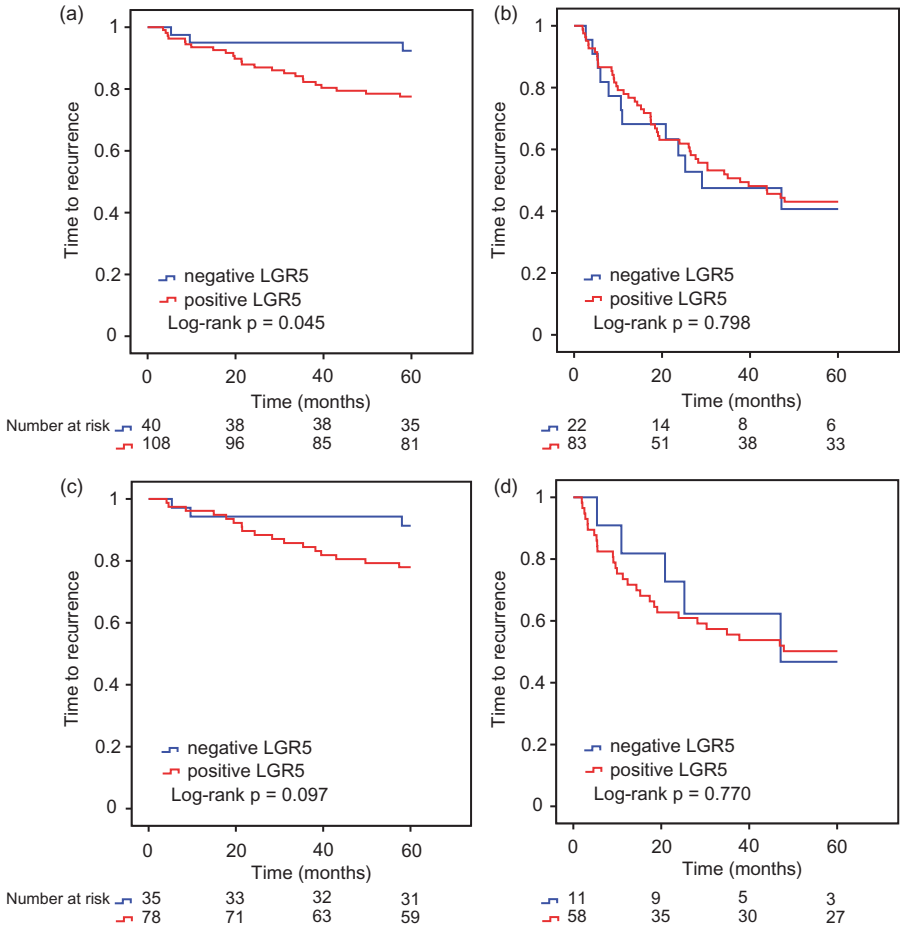

Figure 3. Kaplan-Meier showing 5-year time to recurrence for (a) LGR5 expression in colorectal stage II (b) LGR5 expression in colorectal stage III (c) LGR5 expression in colon stage II (d) LGR5 expression in colon stage III.

Table 3. Selected Cox proportional hazards models for various colorectal cancer cohorts.

			Univariate			Multivariate (n = 120)		
Cohort	Variables	n	HR	95% CI	p	HR	95% CI	p
Colorectal cancer stage II	Age, years	247	1.01	(0.98, 1.05)	0.477	1.03	(0.98, 1.08)	0.230
	Sex (men vs. women)	247	0.66	(0.37, 1.20)	0.174	0.48	(0.20, 1.12)	0.090
	Adjuvant (vs. only surgery)	247	1.08	(0.60, 1.94)	0.790	0.95	(0.41, 2.20)	0.898
	Ki-67 (<40% vs. ≥40%)	203	2.66	(1.34, 5.29)	0.005	3.13	(1.31, 7.49)	0.010
	LGR5 (neg vs. pos)	148	0.31	(0.09, 1.04)	0.057	0.27	(0.08, 0.95)	0.041
						Multivariate (n = 83)		
Colon cancer stage III	Age, years	104	1.01	(0.98, 1.04)	0.755	0.98	(0.95, 1.02)	0.338
	Sex (men vs. women)	104	1.14	(0.66, 1.95)	0.639	0.86	(0.47, 1.56)	0.624
	Adjuvant (vs. only surgery)	104	0.48	(0.28, 0.84)	0.009	0.63	(0.34, 1.15)	0.132
	Ki-67 (<40% vs. ≥40%)	84	1.37	(0.76, 2.49)	0.300	1.54	(0.82, 2.88)	0.179
	CD133(neg vs. pos)	99	1.71	(0.96, 3.02)	0.067	2.18	(1.10, 4.33)	0.026
Predictive analysis						Multivariate (n = 99)		
Colon cancer stage III	Age, years					0.99	(0.96, 1.02)	0.569
	Sex (men vs. women)					0.91	(0.52, 1.60)	0.738
	Adjuvant (vs. only surgery)					0.69	(0.27, 1.77)	0.438
	CD133 (neg vs. pos)					2.29	(1.04, 5.03)	0.040
	CD133 × Adjuvant					0.59	(0.18, 1.89)	0.374

a statistically significant better predictive effect of adjuvant treatment for patients with lack of CD133 expression, but the interaction between CD133 and adjuvant chemotherapy was not statistically significant in multivariate analysis (p = 0.374).

LGR5 ISH was utilized due to the lack of suitable and specific immunohistochemical antibodies against LGR5 [29,30]. LGR5 ISH staining in normal mucosa resembled the staining pattern reported before for intestinal tissue [14,16]. In concordance with the study of Ziskin et al., LGR5 mRNA was upregulated in CRC [16]. One weakness of our study is that we censored 37% of CRC samples due to negative score of the positive control (POLR2A). Nevertheless, the remaining samples were representative of the whole CRC cohort, as demonstrated by the goodness of fit test.

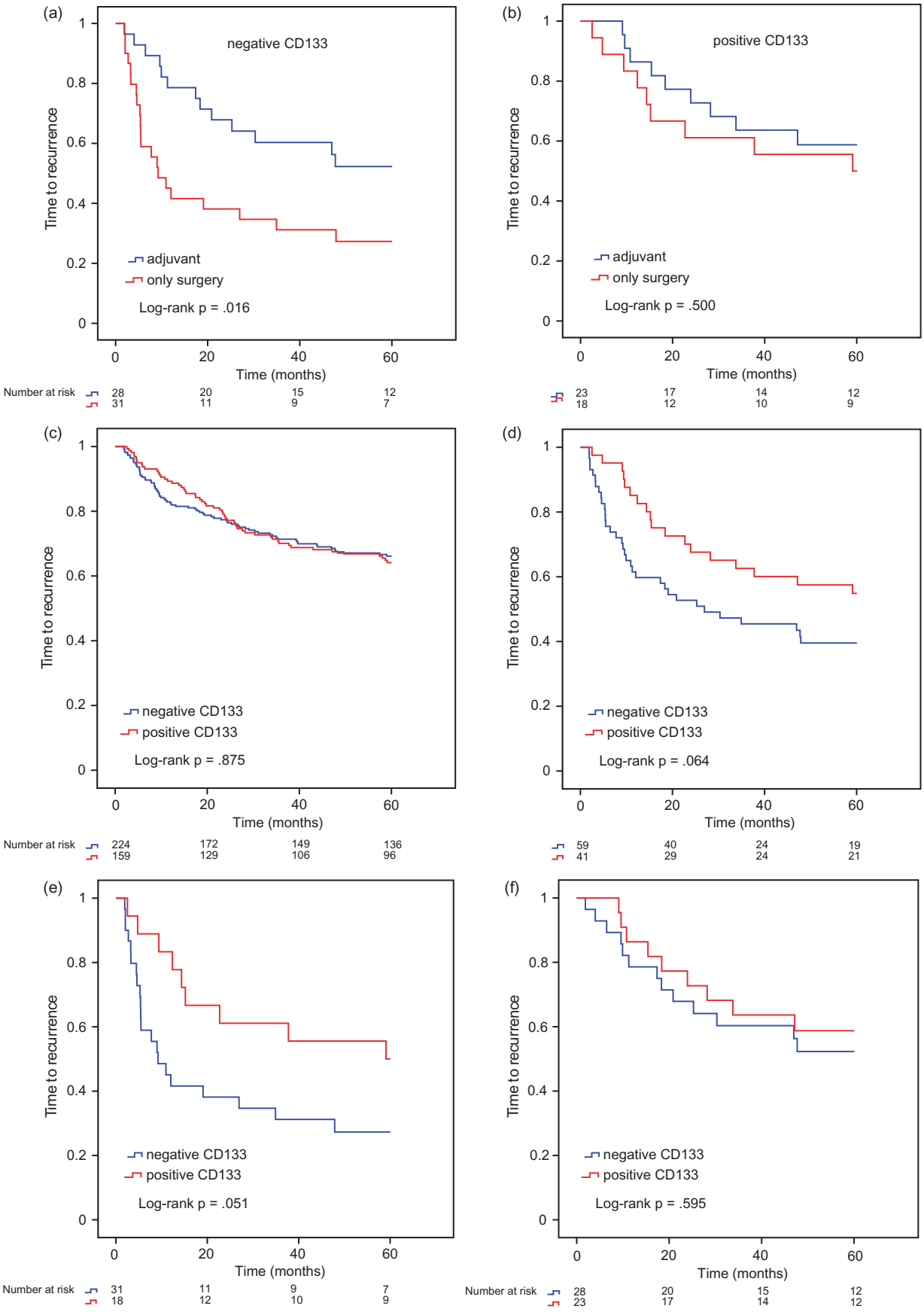

Figure 4. Kaplan–Meier curves showing 5-year time to recurrence for (a) CD133 expression in colorectal cancer (b) CD133 expression in colon stage III (c) CD133 expression in colon stage III who were treated only with surgery (d) CD133 expression in colon stage III who were also treated with adjuvant chemotherapy (e) predictive analysis of the benefit of adjuvant therapy in colon stage III for the group with negative CD133 expression (f) for the group with positive CD133 expression.

Regarding the CD133 protein, at least seven isoforms exist [31]. In addition, the extracellular loops of the protein are glycosylated [32]. Numerous commercially available antibodies targeting different epitopes of the protein contribute to the complexity in published findings on CD133 and which antibody to use is debated [27]. In our study, CD133 exhibited expression on apical/endoluminal membrane of CRC with debris in ductal lumina, a staining pattern described before for this antibody in CRC and pancreatic ductal carcinoma [27,33,34].

In our cohort, there was an association between positive LGR5 mRNA expression and classic adenocarcinoma, but the finding has to be interpreted with caution, as there were only 25 tumors with mucinous and signet-ring cell histological types. There was, however, no association between LGR5 mRNA expression and Ki-67 protein expression. Other groups have identified an association between LGR5 and histological type, depth of invasion, lymph node metastasis, distant metastasis, TNM stage, Ki-67 expression and tumor differentiation in CRC, but the findings are not consistent [16,35–38]. For colon cancer patients in our study, positive LGR5 mRNA expression was associated with TNM stage III and classic adenocarcinoma. The association between positive LGR5 mRNA expression and smaller tumor diameter was observed, both for the CRC stage II and colon cancer stage II cohort. We are not aware of any study, which has identified this inverse correlation between tumor diameter and expression of LGR5 mRNA. Tumor diameter is a controversial marker in CRC [39], but nevertheless, in a study of Kornprat et al., higher tumor diameter was associated with higher TNM T stage and poor progression-free and cancer-specific survival [40].

For all patients in our study, positive CD133 expression correlated with classic adenocarcinoma, higher tumor differentiation and higher Ki-67 expression. In a previous study of our group, high Ki-67 staining was a positive prognostic marker in CRC [26]. In the meta-analysis including 15 studies of immunohistochemical CD133 expression, high CD133 expression correlated with higher TNM T stage, lymph node metastases and vascular invasion in CRC [41]. Ziskin et al. also used ISH instead of IHC for detection of LGR5 mRNA expression in CRC [16]. In the latter study, LGR5 mRNA is not associated with any changes in overall survival. In our study, we have identified an association between positive LGR5 mRNA expression and poorer TTR in CRC stage II cohort. To our knowledge, it is the first study which shows prognostic value of LGR5 mRNA expression for colon cancer stage II, when ISH is utilized.

The meta-analysis also showed that high expression of CD133 is associated with poor overall survival and TTR [41]. Although not statistically significant, the results of Kaplan-Meier analysis in our cohort of colon cancer stage III patients point in the opposite direction. In our multivariate Cox regression model for colon cancer stage III cohort, positive CD133 expression was as a statistically significant prognostic factor for favorable prognosis. The fact that CD133 expression was a prognostic factor for colon cancer stage III patients in multivariate analysis Cox regression model, but not in univariate Cox regression model, may be caused by competing or overlapping variables in the multivariate analysis, most likely it is due to the effect of 5-FU treatment.

In addition, regarding colon cancer stage III patients, better effect of chemotherapy was associated with lack of CD133 expression in Kaplan-Meier analysis. However, the interaction between CD133 expression and treatment was not statistically significant in multivariate analysis. Ong et al. performed their immunohistological analysis with the same antibody as we did; they showed expression of CD133 to be associated with poorer survival in both the surgery alone group and in the chemotherapy group of their CRC cohort, including patients with stage I–IV [22]. Further, for CRC stage III, response to chemotherapy was associated with negative CD133 expression [22]. Thus, their findings for CRC stage III are in line with our finding from Kaplan-Meier analysis.

In conclusion, we have identified LGR5 mRNA expression as prognostic factor for CRC stage II patients, whereas the results of CD133 expression as prognostic and predictive biomarker were inconclusive.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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