

This article is licensed under a Creative Commons Attribution-NonCommercial NoDerivatives 4.0 International License.

A Panel of Tumor Biomarkers to Predict Complete Pathological Response to Neoadjuvant Treatment in Locally Advanced Rectal Cancer

Chiara Dalle Fratte,* Silvia Mezzalana,* Jerry Polesel,† Elena De Mattia,* Antonio Palumbo,‡
Angela Buonadonna,§ Elisa Palazzari,¶ Antonino De Paoli,¶ Claudio Belluco,# Vincenzo Canzonieri,‡**
Giuseppe Toffoli,* and Erika Cecchin*

*Experimental and Clinical Pharmacology, Centro di Riferimento Oncologico di Aviano (CRO) IRCCS, Aviano, Italy

†Epidemiology and Statistics, Centro di Riferimento Oncologico di Aviano (CRO) IRCCS, Aviano, Italy

‡Pathology, Centro di Riferimento Oncologico di Aviano (CRO) IRCCS, Aviano, Italy

§Medical Oncology Unit, Centro di Riferimento Oncologico di Aviano (CRO) IRCCS, Aviano, Italy

¶Radiation Oncology, Centro di Riferimento Oncologico di Aviano (CRO) IRCCS, Aviano, Italy

#Surgical Oncology, Centro di Riferimento Oncologico di Aviano (CRO) IRCCS, Aviano, Italy

**Department of Medical, Surgical and Health Sciences, University of Trieste, Trieste, Italy

Pathological complete response after neoadjuvant chemoradiotherapy in locally advanced rectal cancer patients is related to a favorable prognosis. The identification of early biomarkers predictive of pathological complete response would help optimize the multimodality management of the patients. A panel of 11 tumor-related proteins was investigated by immunohistochemistry in the pretreatment biopsy of a group of locally advanced rectal cancer patients to identify early biomarkers of pathological complete response to neoadjuvant chemoradiotherapy. A mono-institutional retrospective cohort of 95 stage II/III locally advanced rectal cancer patients treated with neoadjuvant chemoradiotherapy and surgery was selected based on clinical–pathological characteristics and the availability of a pretreatment tumor biopsy. Eleven selected protein marker expression (MLH1, GLUT1, Ki67, CA-IX, CXCR4, COX2, CXCL12, HIF1 α , VEGF, CD44, and RAD51) was investigated. The optimal cutoff values were calculated by receiver operating characteristic curve analysis. Classification and regression tree analysis was performed to investigate the biomarker interaction. Patients presenting either Ki-67 or HIF1 α or RAD51 below the cutoff value, or CXCR4 or COX2 above the cutoff value, were more likely to get a pathological complete response. Classification and regression tree analysis identified three groups of patients resulting from the combination of Ki-67 and CXCR4 expression. Patients with high expression of Ki-67 had the lowest chance to get a pathological complete response (18%), as compared to patients with low expression of both Ki-67 and CXCR4 (29%), and patients with low Ki-67 and high CXCR4 expression (70%). Pretreatment Ki-67, CXCR4, COX2, HIF1 α , and RAD51 in tumor biopsies are associated with pathological complete response after neoadjuvant chemoradiotherapy in locally advanced rectal cancer. A combined evaluation of Ki-67 and CXCR4 would increase their predictive potential. If validated, their optimal cutoff could be used to select patients for a tailored multimodality treatment.

Key words: Rectal cancer; Neoadjuvant chemoradiotherapy (nCRT);

Pathological complete response (pCR); Predictive biomarkers; Immunohistochemistry (IHC)

INTRODUCTION

The standard of care for the clinical management of stage II/III locally advanced rectal cancer relies on neoadjuvant chemoradiotherapy treatment, followed by radical surgery including total mesorectal excision, optionally followed by an adjuvant chemotherapy, which was proven to be more effective than surgery alone in terms

of local relapse prevention even if it did not affect overall survival^{1,2}. The pathological examination of surgical specimens represents nowadays the gold standard for the assessment of pathological response to neoadjuvant treatment. A pathological complete response to neoadjuvant chemoradiotherapy, which is the absence of visible residual tumor cells, is commonly observed in a subset of 15% to 30% of locally advanced rectal cancer patients^{3,4}, which

Address correspondence to Prof. Vincenzo Canzonieri, M.D., Director Pathology Unit, Centro di Riferimento Oncologico IRCCS, Via Franco Gallini, 2, 33081 Aviano, Pordenone, Italy. Tel: +39 0434 659618-292; Fax: +39 0434 659370; E-mail: vcanzonieri@cro.it, vcanzonieri@units.it

is associated with longer overall survival and lower risk of local and distant recurrence after surgery with respect to patients with incomplete pathological response⁵.

Organ-sparing strategies such as conservative surgery or watch-and-wait approaches could be considered in patients with clinical complete response to improve quality of life⁶. On the other hand, intensified neoadjuvant programs could be evaluated for poor responders. Therefore, the possibility to predict the outcome of neoadjuvant chemoradiotherapy before treatment or during its very early course would be of crucial clinical relevance in patient risk stratification⁷.

The selection of personalized treatment strategies is currently based essentially on clinical–pathological criteria, including clinical T and N stages, distance of tumor from the anal verge, mesorectal fascia involvement, and extramural vascular invasion. Additional more effective stratification criteria are needed. Major attention was posed on the immunohistochemical expression of proteins with a crucial role in triggering and sustaining tumor cells' growth and proliferation, immune response stimulation, and DNA repair of radiotherapy-related damage.

Several proteins were shown to play a role in modulating the response to chemoradiotherapy in locally advanced rectal cancer, mainly by triggering and sustaining mechanisms of the cellular adaptation to radiotherapy-related damage. However, no final consensus was achieved on their predictive role, and none of them reached enough impact to be considered for the use in the clinical practice⁸.

In the present study, a panel of tumor markers (MLH1, GLUT1, Ki-67, CA-IX, CXCR4, COX2, CXCL12, HIF1 α , VEGF, CD44, and RAD51) belonging to the molecular pathways previously investigated in the context of locally advanced rectal cancer was considered. Those factors were previously reported to have a pivotal role in orchestrating the tumor cells' adaptation to radiotherapy-related damage, impacting the cell cycle^{9,10}, the cells reaction to hypoxia^{11,12}, the mechanisms of DNA mismatch repair¹³, and the inflammation process¹⁴. The selected proteins were investigated by immunohistochemistry in pretreatment biopsies of a group of stage II/III locally advanced rectal cancer patients with the aim to evaluate their association with pathological complete response.

Moreover, the potential interaction among the investigated biomarkers and the patients' clinical–pathological features, as tumor response phenotype, was assessed by a classification and regression tree analysis.

MATERIALS AND METHODS

Patients

The present study includes a retrospective cohort of 95 patients with clinically confirmed stage II–III rectal

adenocarcinoma who were admitted at IRCCS Centro di Riferimento Oncologico di Aviano (Italy) from 2005 to 2014. Patient inclusion criteria were (1) histologically confirmed diagnosis of primary resectable locally advanced rectal cancer by a diagnostic staging colonoscopy, (2) confirmed absence of distant metastases, (3) age ≥ 18 years, (4) stage of disease cT2–cT3–cT4 and N0–N2, (5) performance status (World Health Organization) 0–2, (6) a planned neoadjuvant chemoradiotherapy, and (7) availability of a tumor biopsy sample. The disease extent (T stage), lymph node involvement (N stage), and the presence of visible metastatic lesions (M stage) were assessed by means of magnetic resonance imaging and computed tomography scans. All procedures performed in this study were in accordance with the institutional ethical standards and with the Helsinki Declaration. All the patients provided signed informed consent for research purposes at the time of treatment.

Neoadjuvant Chemoradiotherapy and Surgery

All the patients were treated with neoadjuvant chemoradiotherapy (cumulative radiation dose of 50.4 Gy delivered in 28 daily fractions over a period of 5 weeks). The clinical target volume included the primary tumor, with the mesorectum, and the elective pelvic lymph nodes at risk of tumor involvement. Concomitant 5-fluorouracil (5-FU)-based chemotherapy was delivered to 87 out of 95 (91.6%) patients. All patients underwent surgery 7 to 15 weeks (median: 9 weeks) after completion of neoadjuvant chemoradiotherapy.

Immunohistochemical Analysis

The immunohistochemical analysis was performed on pretreatment tumor biopsies, which were collected during staging colonoscopy and were fixed in 4% formalin and embedded in paraffin wax. Sections 3 μ m thick were stained with hematoxylin and eosin to be reviewed by a trained pathologist (V.C.). For immunohistochemistry, 3- μ m-thick sections were stained on a Dako Omnis platform with the following antibodies: MLH1 (monoclonal, clone M1; Ventana Medical System, Oro Valley, AZ, USA), GLUT1 (polyclonal; Cell Marque, Rocklin, CA, USA), Ki-67 (monoclonal, clone 30-9; Ventana Medical System), CA-IX (monoclonal, clone EP161; Ventana Medical System), CXCR4 (polyclonal; AbCam, Cambridge, UK), COX2 (monoclonal, clone SP21; Cell Marque), CXCL12, HIF1 α (monoclonal, clone H1 α 67; Novus Biological, Centennial, CO, USA), vascular endothelial growth factor (VEGF) (polyclonal; Santa Cruz Biotechnology, Dallas, TX, USA), CD44 (monoclonal, clone SP37; Ventana Medical System), and RAD51 (polyclonal; Santa Cruz, Biotechnology). Immunoreactions were developed using 0.03% 3,3'-diaminobenzidine tetrahydrochloride and results that were independently reviewed by two trained

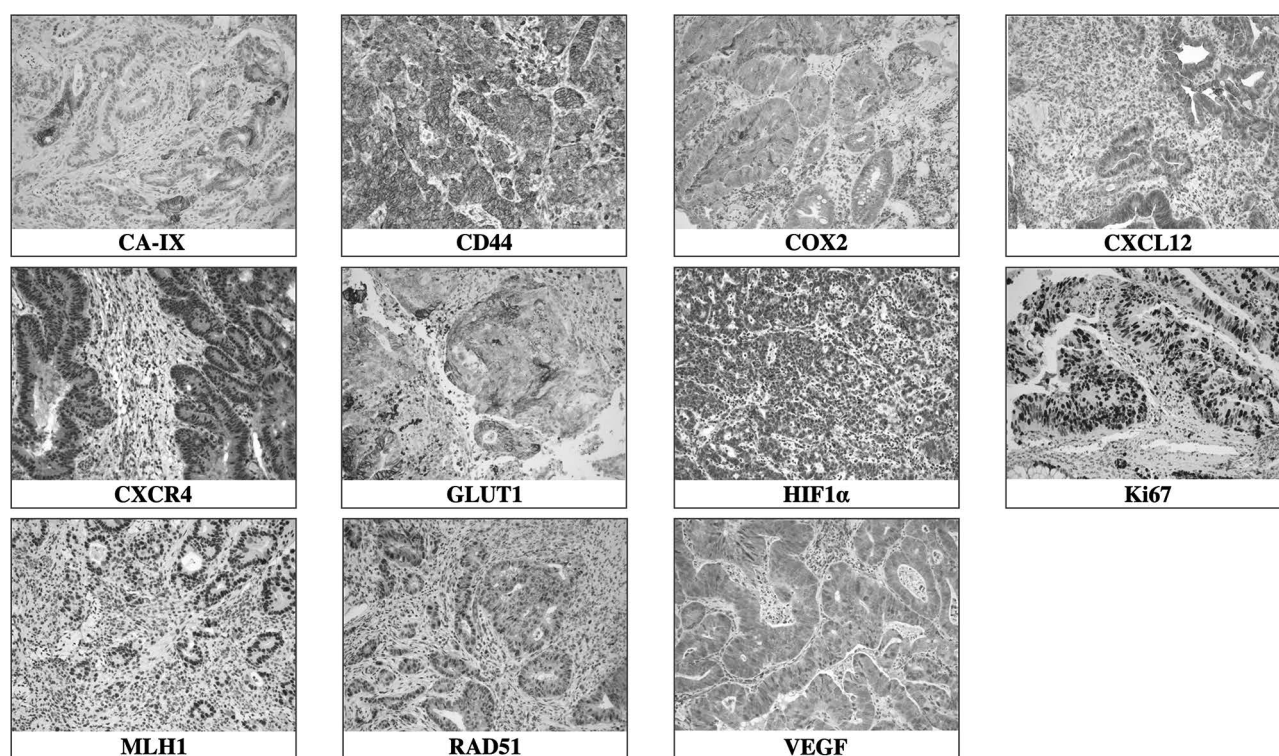


Figure 1. Immunohistochemical reactivity in pretreatment biopsies showing cytoplasmic, nuclear, and membrane reactivity for all the protein biomarkers investigated.

pathologists who were blinded to patients' clinical information and tumor regression grade (TRG) (V.C. and A.P.). Immunostaining was evaluated at the nuclear level for MLH1, Ki-67, and RAD51; at the membrane level for CA-IX and CD44; at the cytoplasmic level for GLUT1, COX2, CXCL12, HIF1 α , and VEGF; and at the nuclear and cytoplasmic level for CXCR4 (Fig. 1). Expression of proteins was assessed by evaluating the intensity of staining (0, absent; 1, weak; 2, moderate; and 3, strong) and the proportion of cells presenting nuclear, cytoplasmic, or membrane staining positivity (ranging from 0% to 100%). The comprehensive immunoreactivity score (H-score) was calculated using a widely accepted semiquantitative method¹⁵. Briefly, the percentage of positive cells was ranked into five categories (0: 0% of positive cells; 1: 1%–24% of positive cells; 2: 25%–49% of positive cells; 3: 50%–74% of positive cells; and 4: 75%–100% of positive cells) according to the fraction of cells exhibiting staining positivity. The H-score was then derived by multiplying the ranked percentage of cells presenting immunostaining positivity by the staining intensity. H-score values ranged from 0 to 12.

Assessment of Response to Neoadjuvant Chemoradiotherapy and Patient Follow-Up

Pathological staging was reported following the UICC TNM Classification of Malignant Tumours (8th ed.)¹⁶.

The pathological tumor response to neoadjuvant chemoradiotherapy was adapted from the TRG criteria proposed by Mandard et al.¹⁷. All patients were followed up after treatment every 3 months for the first 2 years, every 6 months thereafter up to 5 years, and then yearly.

Statistical Analysis

Pathological tumor response to neoadjuvant chemoradiotherapy was defined according to TRG. Complete responders (TRG1) were compared to non-complete responders (TRG2–4). The biomarkers' expression level was considered as a continuous variable; considering the non-normal distribution, differences between TRG1 and TRG2–4 patients were evaluated nonparametrically through the Mann–Whitney test. For each biomarker, a receiver operating characteristic (ROC) analysis was performed to select the optimal cutoff level for response prediction; each biomarker was then dichotomized according to its optimal cutoff. The risk of complete response [odds ratio (OR)] and corresponding 95% confidence intervals (CIs) were estimated by applying a multivariable unconditional logistic regression model, adjusting for cN stage at the diagnosis, distance from anal verge (<7 cm), and neoadjuvant chemotherapy scheme (5-FU-based alone, 5-FU-based in combination, and none).

To evaluate the potential interaction between biomarkers, a classification and regression tree analysis was used to predict TRG1. The classification and regression tree is the result of a recursive partitioning procedure that creates subsets starting from the entire dataset. Initially, the procedure splits the entire dataset using the variable—among all considered predictors—that is associated the most with TRG1. This process is repeated on each derived subset in a recursive manner, stopping when splitting no longer adds value to the classification. Since a multiparameter scoring system was used to rate the markers' expression, the semiquantitative approach (H-score) was used to perform the classification and regression tree. Patients were then categorized according to the classification and regression tree subgroups, and ORs for TRG1 were calculated for each subgroup. All statistical analyses were performed using SAS software (SAS Institute Inc., Cary, NC, USA) and R 3.9. Values of $p < 0.05$ (two-sided) were considered statistically significant.

RESULTS

Patients' Characteristics and Response to Neoadjuvant Chemoradiotherapy

Clinical characteristics and treatment description of the 95 patients selected are listed in Table 1. In detail, all the 95 patients received a radiotherapy treatment. Of those, 45 patients out of 95 (47.4%) were cotreated with fluoropyrimidine monotherapy, and 41 of 95 (43.2%) received a fluoropyrimidine in combination with other drugs. Drugs administered in association were oxaliplatin ($n = 27$), gefitinib ($n = 9$), raltitrexed ($n = 4$), and irinotecan ($n = 1$). Patients administered with gefitinib and raltitrexed were enrolled in specific clinical trials^{18,19}. Nine patients out of 95 (9.5%) did not receive chemotherapy in concomitance to radiotherapy. After completing neoadjuvant chemoradiotherapy, 25 of 95 (26.3%) patients achieved a pathological complete response (ypT0N0) (responders), while 70 of 95 (73.7%) patients reported a partial or null tumor response (nonresponders). No patient reported a TRG5. With a median follow-up of 53.2 months (range: 2–147), the local and distant 3-year cumulative rates were 13.1% (95% CI: 6.9%–21.3%) and 29.0% (95% CI: 19.8%–38.8%), respectively, whereas the 3-year overall survival was 92.9%.

Association Between Immunohistochemistry Biomarker Expression and Tumor Response to Neoadjuvant Chemoradiotherapy

Table 2 reports the expression level of each protein in responder and nonresponder patients. TRG1 patients showed a significantly higher CXCR4 and COX2 H-score (increased expression) than those with TRG2–4 (CXCR4, H-score: 3 vs. 2, $p = 0.010$; COX2, H-score: 6 vs. 4, $p = 0.030$). When considering cellularity and

immune-staining intensity parameters for these same markers, we observed that the median cellularity was similar between responders and nonresponders, with only a weak tendency for CXCR4 cellularity to be higher among responders (35% vs. 20%, $p = 0.188$). Differences between TRG1 and TRG2–4 patients emerged also for staining intensity: indeed, the proportion of patients with moderate/strong intensity was 59.1% and 24.6%, respectively, for CXCR4 ($p = 0.002$), and 68.0% and 41.4% for COX2 ($p = 0.042$).

Table 3 reports the optimal cutoff values according to ROC analysis to discriminate between responders and nonresponders. The multivariate OR to get a pathological complete response to neoadjuvant chemoradiotherapy was calculated for each marker based on those cutoff values.

Significant association with the probability of TRG1 emerged for Ki-67, CXCR4, COX2, HIF1 α , and RAD51. Among the 34 patients showing low expression of Ki-67 (H-score < 7), 14 (41.1%) were responders, while only 11 (18.0%) patients among those with high expression

Table 1. Patient and Treatment Characteristics

Characteristic	N (%)
All	95 (100%)
Median age at diagnosis, years (range)	65 (25–85)
Gender	
Male	68 (71.6%)
Female	27 (28.4%)
Median distance from anal verge [cm (range)]	6 (2–12)
Imaging pretreatment staging (TNM)	
cT2N+	6 (6.3%)
cT3N0	21 (22.1%)
cT3N+	67 (70.5%)
N.A.	1 (1.1%)
Tumor regression grade (by Mandard's)	
1	25 (26.3%)
2	13 (13.7%)
3	43 (45.3%)
4	14 (15.6%)
Type of surgery	
Low anterior resection (LAR)	57 (60.0%)
Local excision (LE)	13 (13.7%)
Transanal local excision (TALE)	5 (5.3%)
Transanal endoscopic microsurgery (TEM)	4 (4.2%)
Others	16 (16.8%)
Radiotherapy	
≤ 5040 cGy	87 (91.6%)
> 5040 cGy	8 (8.4%)
Chemotherapy	
Fluoropyrimidines monotherapy	45 (47.4%)
Fluoropyrimidines + other*	41 (43.2%)
No chemotherapy	9 (9.5%)

*Oxaliplatin ($N = 27$), gefitinib ($N = 9$), raltitrexed ($N = 4$), and irinotecan ($N = 1$).

Table 2. Median Value and Interquartile Range (Q1–Q3) of H-Score and Neoplastic Cellularity and Prevalence of Moderate/Strong Intensity for Selected Parameters According to TRG Status

	Patients		H-Score (Huang F)			Cellularity (%)			Moderate/Strong Intensity		
	TRG1	TRG2–4	TRG1	TRG2–4	<i>p</i> Value*	TRG1	TRG2–4	<i>p</i> value*	TRG1	TRG2–4	<i>p</i> Value†
MLH1	25	62	6 (1–12)	8 (4–12)	<i>p</i> = 0.126	60 (20–90)	80 (40–90)	<i>p</i> = 0.221	64.0%	77.4%	<i>p</i> = 0.430
GLUT 1	25	70	6 (4–8)	6 (3–9)	<i>p</i> = 0.903	50 (30–60)	60 (40–70)	<i>p</i> = 0.331	84.0%	82.9%	<i>p</i> = 0.864
Ki-67	25	70	6 (3–9)	9 (6–9)	<i>p</i> = 0.059	40 (20–70)	60 (40–70)	<i>p</i> = 0.070	100%	100%	<i>p</i> = 1.000
CA IX	25	70	2 (1–2)	2 (0–2)	<i>p</i> = 0.555	5 (2–20)	10 (0–20)	<i>p</i> = 0.721	52.0%	48.6%	<i>p</i> = 0.786
CXCR4	20	61	3 (2–5)	2 (1–3)	<i>p</i> = 0.010	35 (20–50)	20 (10–50)	<i>p</i> = 0.188	59.1%	24.6%	<i>p</i> = 0.002
COX2	25	70	6 (3–8)	4 (3–6)	<i>p</i> = 0.030	70 (60–80)	70 (50–80)	<i>p</i> = 0.649	68.0%	41.4%	<i>p</i> = 0.042
CXCL12	25	70	2 (2–6)	3 (1–6)	<i>p</i> = 0.778	30 (20–50)	30 (10–50)	<i>p</i> = 0.861	64.0%	55.7%	<i>p</i> = 0.067
HIF1 α	25	70	4 (2–6)	6 (2–8)	<i>p</i> = 0.345	70 (40–80)	70 (40–80)	<i>p</i> = 0.910	36.0%	55.7%	<i>p</i> = 0.122
VEGF	25	70	2 (1–3)	3 (1–6)	<i>p</i> = 0.179	40 (10–60)	60 (20–80)	<i>p</i> = 0.081	16.0%	28.6%	<i>p</i> = 0.478
CD44	25	70	6 (6–8)	6 (4–8)	<i>p</i> = 0.608	60 (50–80)	70 (40–70)	<i>p</i> = 0.799	92.0%	84.3%	<i>p</i> = 0.848
RAD51	25	68	4 (1–6)	4 (2–6)	<i>p</i> = 0.254	45 (20–50)	50 (30–65)	<i>p</i> = 0.177	59.1%	74.6%	<i>p</i> = 0.350

*Mann–Whitney test; †Fisher's exact test.

of Ki-67 (H-score ≥ 7) were responders (OR for TRG1 = 3.30; 95% CI: 1.19–9.13). Similarly, HIF1 α H-score < 5 was associated with a favorable pathological complete response than high levels (OR = 2.91; 95% CI: 1.01–8.40), as well as RAD51 H-score < 2 (OR = 3.87; 95% CI: 1.05–14.2). By contrast, high expressions of CXCR4 and COX2 were significant predictors of TRG1 (OR = 4.47; 95% CI: 1.15–17.4 for CXCR4 H-score ≥ 2 , and OR = 3.21; 95% CI: 1.14–9.09 for COX2 H-score ≥ 6).

When looking at the association of immunohistochemistry cellularity and immunostaining intensity separately, we observed that the association between the Ki-67 H-score and TRG1 was driven by the cellularity parameter, which permitted to discriminate responders from nonresponders with a greater specificity than the H-score (81.4% vs. 71.4%). All TRG1 and TRG2–4 patients reported moderate/strong Ki-67 staining intensity. For CXCR4, the staining intensity alone, compared to H-score, allowed the discrimination of responders with a higher specificity (75.4% vs. 45.9%, respectively) but a lower sensitivity (60.0% vs. 85.0%, respectively); for COX2, the stain intensity underlined a lower specificity (58.6% vs. 61.4%) and a higher sensitivity (68.0% vs. 64.0%) than the H-score. Nine out of 95 (9.5%) patients did not receive chemotherapy along with radiotherapy. A sensitivity analysis, conducted excluding the nine patients who did not undergo chemotherapy, did not show substantial changes in the results. In particular, the risk of TRG1 was 3.30 (95% CI: 1.01–8.87) for Ki-67 H-score < 7 , 6.50 (95% CI: 1.31–32.12) for CXCR4 H-score ≥ 2 , and 4.41 (95% CI: 1.40–13.87) for Ki-67 cellularity ≤ 0.30 .

Classification and Regression Tree Analysis

A classification and regression tree analysis, including all the protein H-scores and clinical and demographic variables, was performed on the 95-patient study cohort

for the prediction of pathological complete response. The resulting tree (Fig. 2) identified two markers (Ki-67 and CXCR4) to classify the patients into three groups with different risks of getting a TRG1. A Ki-67 H-score ≥ 8 alone identified the patients with the lowest chance of getting TRG1. Among patients with Ki-67 H-score < 8 , those with CXCR4 < 4 reported a risk of getting TRG1 of 1.85 (95% CI: 0.57–5.97), whereas those with CXCR4 H-score ≥ 4 represent the group with the highest percentage of complete responders (70%; OR = 13.49; 95% CI: 2.64–68.99).

DISCUSSION

The identification of early molecular markers predictive of neoadjuvant chemoradiotherapy outcome in locally advanced rectal cancer is being investigated with compelling interest, but results remain questionable and frequently conflicting. We evaluated the immunohistochemistry expression of 12 candidate proteins with relevant biological implication in locally advanced rectal cancer to identify their potential application as predictive markers of pathological complete response. For five markers (Ki-67, CXCR4, COX-2, HIF1 α , and RAD51), we identified a cutoff value of protein expression that could successfully discriminate patients achieving a pathological complete response from nonresponder patients.

Ki-67 is a well-known proliferation marker whose overexpression is commonly recognized as a marker of highly malignant phenotypes in several types of tumors^{20,21}. Accordingly, in our study cohort, patients with a high Ki-67 level were more likely to get a bad tumor response after neoadjuvant chemoradiotherapy. A similar trend was previously reported by Jakob et al., who compared the Ki-67 protein levels in pre- and posttreatment locally advanced rectal cancer biopsies and demonstrated that its overexpression at any time point is an early

Table 3. Odds Ratio (OR) and 95% Confidence Interval (CI)* for TRG1 in 95 Patients With Locally Advanced Rectal Cancer

	H-Score (Huang F)				Cellularity (%)				Intensity			
	Prevalence		Prevalence		Prevalence		Prevalence		Prevalence		Prevalence	
	Cutoff†	TRG1	TRG2-4	OR (95% CI)	Cutoff†	TRG1	TRG2-4	OR (95% CI)	Cutoff	TRG1	TRG2-4	OR (95% CI)
MLH1	<5	48.0%	27.4%	2.62 (0.93–7.39)	≤50	48.0%	258%	2.79 (0.98–7.92)	M/S	64.0%	77.4%	0.52 (0.17–1.60)
GLUT 1	<3	16.0%	7.1%	1.84 (0.37–9.27)	≤50	60.0%	45.7%	1.74 (0.64–4.76)	M/S	84.0%	82.9%	1.26 (0.35–4.62)
Ki-67	<7	56.0%	28.6%	3.30 (1.19–9.13)	≤30	48.0%	18.6%	4.27 (1.47–12.5)	M/S	100%	100%	–
CA IX	≥1	84.0%	74.3%	2.23 (0.62–7.98)	≤5	56.0%	48.6%	1.22 (0.46–3.29)	M/S	52.0%	48.6%	1.33 (0.49–3.63)
CXCR4	≥2	85.0%	54.1%	4.47 (1.15–17.4)	≥20	81.8%	59.0%	3.08 (0.89–10.6)	M/S	59.1%	24.6%	6.08 (1.98–18.7)
COX2	≥6	64.0%	38.6%	3.21 (1.14–9.09)	≤80	92.0%	82.9%	2.00 (0.38–10.5)	M/S	68.0%	41.4%	3.29 (1.15–9.43)
CXCL12	<3	60.0%	48.6%	1.80 (0.67–4.85)	≤30	68.0%	52.9%	2.35 (0.81–6.78)	M/S	64.0%	55.7%	1.20 (0.45–3.23)
HIF1α	<5	68.0%	44.3%	2.91 (1.01–8.40)	≤40	32.0%	27.1%	1.07 (0.36–3.18)	M/S	36.0%	55.7%	0.43 (0.15–1.21)
VEGF	<7	96.0%	80.0%	4.60 (0.55–38.7)	≤60	84.0%	58.6%	2.86 (0.84–9.73)	M/S	16.0%	28.6%	0.63 (0.18–2.19)
CD44	<7	68.0%	64.3%	1.42 (0.49–4.06)	≤60	52.0%	44.3%	1.60 (0.58–4.39)	M/S	92.0%	84.3%	2.19 (0.41–11.6)
RAD51	<2	27.3%	10.3%	3.87 (1.05–14.2)	≤60	95.5%	75.0%	7.18 (0.82–63.2)	M/S	59.1%	74.6%	0.60 (0.20–1.78)

*Adjusted for cN (0 and 1+), distance from anal margin <7 cm, and neoadjuvant chemotherapy (none, 5-FU, and 5-FU + other). †Estimated through ROC analysis based on TRG1.

marker of poor tumor regression¹⁰. However, other studies showed that a higher rate of Ki-67-positive cells in treatment-naïve locally advanced rectal cancer biopsies was associated with a greater incidence of pathological complete response^{22,23}. Therefore, despite an acknowledged bad prognostic value of the marker²⁴, its predictive role on the neoadjuvant chemoradiotherapy in locally advanced rectal cancer is still not elucidated. In our study, Ki-67 was shown to significantly interact with CXCR4, which notably characterizes highly proliferating tumor cells, to discriminate responders from nonresponders by a classification and regression tree analysis. The complexity of the tumor response phenotype should be probably studied with an integrated approach^{10,25}.

In our study, the tumor expression of the chemokine receptor CXCR4 was found to be increased in patients getting a pathological complete response. Despite some data available on CXCR4 prognostic effect²⁶, it was poorly investigated for its role in contributing the sensitivity toward neoadjuvant chemoradiotherapy in locally advanced rectal cancer. It is reasonable to assume that the high proliferation rate of cells overexpressing CXCR4 might increase the local effectiveness of chemoradiation treatments. A recent study, which investigated the predictive role of CXCR4 expression in 85 locally advanced rectal cancer patients before neoadjuvant chemoradiotherapy, highlighted that, besides its expression level, an important role is played by its cellular localization, with the nuclear, or combined cytoplasmic and nuclear localization, related to the greater chance of tumor response²⁷. This is consistent with what we observed in our cases, where high expressing tumors presented a combined nuclear and cytoplasmic intense staining. Further investigations are probably needed to shed light upon the biological interplay between CXCR4-mediated pathways at the cellular level, the tumor response to neoadjuvant chemoradiotherapy, and its predictive role.

Our results support a predictive potential role also for COX2 that appears to be associated with a higher chance of pathological complete response when expressed above the herein defined cutoff value. Despite the well-accepted role of COX2 in supporting tumor growth and development²⁸, literature data are conflicting regarding its predictive significance in locally advanced rectal cancer, with some studies sustaining^{29–31} and others disproving^{32,33} a COX2 involvement in predicting the neoadjuvant chemoradiotherapy efficacy. These discrepancies might be partially attributable to the heterogeneities of study cohorts and/or therapeutic schemes as well as to the huge methodological heterogeneity in the scoring system used to classify COX2 expression. Consistently with other investigators³⁴, we classified tumors as COX2 overexpressing when the immunostaining intensity was defined by the pathologists as “strong” to “moderate.” However, when

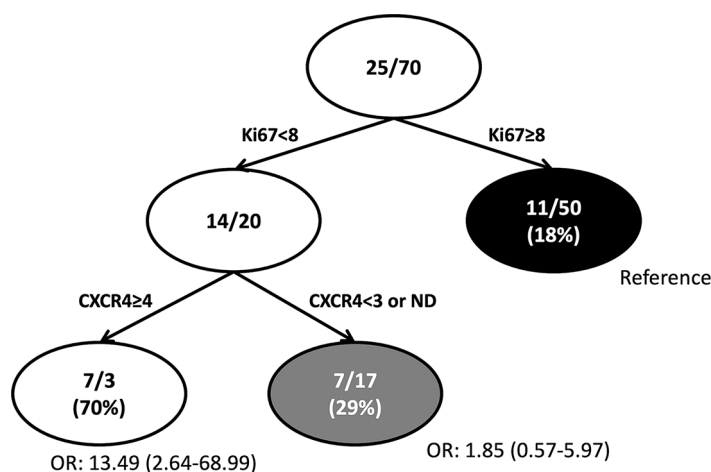


Figure 2. Classification and regression tree representation of the biomarkers' expression combination significantly predictive of pathological complete response (TRG1) in locally advanced rectal cancer patients. Fractions indicate the number of patients reporting a pathological complete response versus patient reporting an incomplete pathological response (TRG2–4). Black circles represent terminal nodes with low probability to report a pathological complete response (ratio <20%); gray circles represent terminal nodes with intermediate probability to report a pathological complete response (20% ≤ ratio < 70%); and white circles represent terminal nodes with low probability to report a TRG2–4 (ratio ≥70%). OR and 95% CI were calculated for each group with respect to the reference group (lower probability) through logistic regression model.

looking at the rate of COX2-positive cells, we noticed that tumors with an extremely high percentage of expressing cells (>80%) were more likely to get a bad tumor response to treatment (data not shown). While the research on the mechanism by which COX2 modulates the sensitivity to neoadjuvant chemoradiotherapy in locally advanced rectal cancer remains a matter of open investigation, our data lead to reconsider its predictive significance as an early biomarker of treatment outcome.

In our cohort, HIF1 α as well as RAD51 overexpression, assessed by means of the H-score and based on specific cutoff values, were associated with a bad tumor response. In pancreatic cancer cells, RAD51 was proven to decrease intracellular reactive oxygen species production and increase the HIF1 α protein level³⁵. Consistently with the biological connection between RAD51 and HIF1 α , our results sustain their matched clinical value as early predictors of poor treatment outcome. Specifically, RAD51 plays an essential role in DNA repair via homologous recombination, and many studies have suggested that the RAD51 expression increased cellular resistance to chemotherapy and radiotherapy³⁶. HIF1 α plays a key role in the cellular adaptation to hypoxia. A few studies reported conflicting findings upon the predictive role of HIF1 α in pretreatment locally advanced rectal cancer biopsies. As reported above for COX2, this heterogeneity could be driven by different technical approaches to the protein expression quantification methods. In a cohort of 86 locally advanced rectal cancer patients, Havelund et al. showed that the HIF1 α expression, measured by means of a semiquantitative score, has no predictive impact on the

response to chemoradiotherapy³⁷. Similarly, Shioya et al., who quantified the percentage of HIF1 α -positive cells in 50 locally advanced rectal cancer patients, did not find significant associations with the pathological grading or pathological complete response³⁸. The semiquantitative scoring system (H-score) we applied in our study, which couples the fraction of positive cells with their staining intensity, possibly allows a more comprehensive assessment of HIF1 α expression and could have helped to highlight previously overlooked associations.

In the framework of complex phenotypic traits, it is of crucial importance to define the mutual interaction between the different players in driving the clinical phenotype. As mentioned above, we exploited a classification and regression tree analysis to put together clinical variables and biomarker expression and found that the combination of Ki-67 and CXCR4 expression assessment enabled the stratification of locally advanced rectal cancer patients into three distinct categories according to response to treatment. A correlation between the level of CXCR4 and Ki-67 mutual expression was reported in other cancers^{39,40}, supporting their cross-interaction in defining the proliferative and metastatic cells phenotype. However, despite the reported biological interplay between Ki-67 and CXCR4, their expression levels were proven to change considerably depending on the location of the primary tumor⁴¹, raising the need for devoted investigations focused on locally advanced rectal cancer.

The main limitations of the present work are the narrow patient cohort and the heterogeneity of the administered treatment. However, a sensitivity analysis, excluding the

patients who received radiotherapy only, did not highlight any significant difference in the association between the selected markers and TRG1. This possibly sustains that the tumor expression of Ki-67 and CXCR4 is involved in the modulation of the response to radiotherapy and that its predictive effect is only minimally affected by the coadministered chemotherapy. The availability of matched surgical tissue to assess the dynamics of protein biomarker levels before and after neoadjuvant chemoradiotherapy might have provided more accurate information upon the biological involvement of the protein markers in tumor cells while on treatment. In addition, the possibility to perform additional molecular analyses (i.e., mRNA expression analysis) would have provided a validation of our results. Unfortunately, the retrospective study design prevented the collection of suitable tumor material.

CONCLUSIONS

In conclusion, the present work identified five protein markers (Ki-67, CXCR4, COX2, HIF1 α , and RAD51) that, when measured in pretreatment biopsies, can discriminate responder and nonresponder locally advanced rectal cancer patients. For each one of them, we calculated the best fitting expression cutoff value with the maximal sensitivity and specificity. Moreover, we underscored a mutual correlation between Ki-67 and CXCR4 in defining different risk categories according to their relative expression levels. The early identification of patients more likely to get a pathological complete response from a neoadjuvant chemoradiotherapy could be helpful to improve the multimodality treatment refinement, an emergent issue in locally advanced rectal cancer patients, but further prospective clinical trials are warranted to clarify its clinical utility in the framework of rectal cancer.

ACKNOWLEDGMENTS: *The authors acknowledge Dr. Paola Ceolin and Dr. Elisa Comaro for their valuable technical support in the immunohistochemical analyses. The authors declare no conflicts of interest.*

REFERENCES

1. Bosset J-F, Collette L, Calais G, Mineur L, Maingon P, Radošević-Jelic L, Daban A, Bardet E, Beny A, Ollier J-C. Chemotherapy with preoperative radiotherapy in rectal cancer. *N Engl J Med*. 2006;355(11):1114–1123.
2. van Gijn W, Marijnen CA, Nagtegaal ID, Kranenbarg EM-K, Putter H, Wiggers T, Rutten HJ, Pahlman L, Glimelius B, van de Velde CJ. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer: 12-Year follow-up of the multicentre, randomised controlled TME trial. *Lancet Oncol*. 2011;12(6):575–582.
3. Maas M, Nelemans PJ, Valentini V, Das P, Rödel C, Kuo L-J, Calvo FA, García-Aguilar J, Glynne-Jones R, Haustermans K, Mohiuddin M, Pucciarelli S, Small WJ, Theodoropoulos G, Biondo S, Beets-Tan RG, Beets GL. Long-term outcome in patients with a pathological complete response after chemoradiation for rectal cancer: A pooled analysis of individual patient data. *Lancet Oncol*. 2010;11(9):835–844.
4. Sanghera P, Wong DWY, McConkey CC, Geh JI, Hartley A. Chemoradiotherapy for rectal cancer: An updated analysis of factors affecting pathological response. *Clin Oncol*. 2008;20(2):176–183.
5. Zorcolo L, Rosman AS, Restivo A, Pisano M, Nigri GR, Fancellu A, Melis M. Complete pathologic response after combined modality treatment for rectal cancer and long-term survival: A meta-analysis. *Ann Surg Oncol*. 2012;19(9):2822–2832.
6. Pang K, Rao Q, Qin S, Jin L, Yao H, Zhang Z. Prognosis comparison between wait and watch and surgical strategy on rectal cancer patients after treatment with neoadjuvant chemoradiotherapy: A meta-analysis. *Therap Adv Gastroenterol*. 2019;12:175628481989247.
7. Renehan AG, Malcomson L, Emsley R, Gollins S, Maw A, Myint AS, Rooney PS, Susnerwala S, Blower A, Saunders MP, Wilson MS, Scott N, O'Dwyer S. Watch-and-wait approach versus surgical resection after chemoradiotherapy for patients with rectal cancer (the OnCoRe project): A propensity-score matched cohort analysis. *Lancet Oncol*. 2016;17(2):174–183.
8. Schmoll HJ, Van Cutsem E, Stein A, Valentini V, Glimelius B, Haustermans K, Nordlinger B, van de Velde CJ, Balmana J, Regula J, Nagtegaal ID, Beets-Tan RG, Arnold D, Ciardiello F, Hoff P, Kerr D, Köhne CH, Labianca R, Price T, Scheithauer W, Sorbero A, Tabernero J, Aderka D, Barroso S, Bodoky G, Douillard JY, El Ghazaly H, Gallardo J, Garin A, Glynne-Jones R, Jordan K, Meshcheryakov A, Papamichail D, Pfeiffer P, Souglakos I, Turhal S, Cervantes A. ESMO Consensus Guidelines for management of patients with colon and rectal cancer: A personalized approach to clinical decision making. *Ann Oncol*. 2012;23(10):2479–2516.
9. Brown JR, DiGiovanna MP, Killelea B, Lannin DR, Rimm DL. Quantitative assessment Ki-67 score for prediction of response to neoadjuvant chemotherapy in breast cancer. *Lab Invest*. 2014;94(1):98–106.
10. Jakob C, Liersch T, Meyer W, Becker H, Baretton GB, Aust DE. Neuro-regulation of lower esophageal sphincter predictive value of Ki67 and p53 in locally advanced rectal cancer: Correlation with thymidylate synthase and histopathological tumor regression after neoadjuvant 5-FU-based chemoradiotherapy. *World J Gastroenterol*. 2008;14(7):1060.
11. Horsman MR, Mortensen LS, Petersen JB, Busk M, Overgaard J. Imaging hypoxia to improve radiotherapy outcome. *Nat Rev Clin Oncol*. 2012;9(12):674–687.
12. Vitoratou I, Tolia M, Liakos P, Tsoukalas N, Giaginis C, Nikolaou M, Nikolaou G, Rigas G, Lioupis A, Kyrgias G. Clinical value of significance of hypoxia inducible factor-1 α , glucose transporter-1 and carbonic anhydrase IX in rectal cancer after preoperative chemoradiotherapy. *J BUON*. 2019;24(2):456–463.
13. Ostwal V, Pande NS, Engineer R, Saklani A, deSouza A, Ramadwar M, Sawant S, Mandavkar S, Shrirangwar S, Kataria P, Patil P, Shetty O, Ramaswamy A. Low prevalence of deficient mismatch repair (dMMR) protein in locally advanced rectal cancers (LARC) and treatment outcomes. *J Gastrointest Oncol*. 2018;10(1):19–29.
14. Shin YK, Park JS, Kim HS, Jun HJ, Kim GE, Suh CO, Yun YS, Pyo H. Radiosensitivity enhancement by celecoxib, a cyclooxygenase (COX)-2 selective inhibitor, via COX-2-dependent cell cycle regulation on human cancer cells expressing differential COX-2 levels. *Cancer Res*. 2005;65(20):9501–9509.

15. Huang D, Sun W, Zhou Y, Li P, Chen F, Chen H, Xia D, Xu E, Lai M, Wu Y, Zhang H. Mutations of key driver genes in colorectal cancer progression and metastasis. *Cancer Metastasis Rev.* 2018;37(1):173–187.
16. James D, Brierley, Mary K, Gospodarowicz, Christian Wittekind. *TNM classification of malignant tumours*, 8th ed. Oxford (UK)/Hoboken (NJ): John Wiley & Sons, Inc; 2017. p. 79–88.
17. Mandard A-M, Dalibard F, Mandard J-C, Marnay J, Henry-Amar M, Petiot J-F, Roussel A, Jacob J-H, Segol P, Samama G, Ollivier J-M, Bonvalot S, Gignoux M. Pathologic assessment of tumor regression after preoperative chemoradiotherapy of esophageal carcinoma. *Clinicopathologic correlations.* *Cancer* 1994;73(11):2680–2686.
18. Gambacorta MA, De Paoli A, Lupattelli M, Chiloiro G, Solazzo AP, Barbaro B, Alfieri S, Vecchio FM, Lenkowicz J, Navarria F, Palazzari E, Bertola G, Frattegiani A, Minsky B, Valentini V. Phase I and II trial on infusional 5-fluorouracil and gefitinib in combination with preoperative radiotherapy in rectal cancer: 10-Years median follow-up. *Clin Transl Radiat Oncol.* 2018;10:23–28.
19. Gambacorta MA, Valentini V, Morganti AG, Mantini G, Miccichè F, Ratto C, Di Miceli D, Rotondi F, Alfieri S, Doglietto GB, Vargas JG, De Paoli A, Rossi C, Cellini N. Chemoradiation with raltitrexed (TOMUDEX) in preoperative treatment of stage II-III resectable rectal cancer: A phase II study. *Int J Radiat Oncol Biol Phys.* 2004;60(1):130–138.
20. Yerushalmi R, Woods R, Ravdin PM, Hayes MM, Gelmon KA. Ki67 in breast cancer: Prognostic and predictive potential. *Lancet Oncol.* 2010;11(2):174–183.
21. Zhou Y, Hu W, Chen P, Abe M, Shi L, Tan S, Li Y, Zong L. Ki67 is a biological marker of malignant risk of gastrointestinal stromal tumors: A systematic review and meta-analysis. *Medicine (Baltimore)* 2017;96(34):e7911.
22. Kim NK, Park JK, Lee KY, Yang WI, Yun SH, Sung J, Min JS. p53, BCL-2, and Ki-67 expression according to tumor response after concurrent chemoradiotherapy for advanced rectal cancer. *Ann Surg Oncol.* 2001;8(5):418–424.
23. Hur H, Kim NK, Min BS, Baik SH, Lee KY, Koom WS, Ahn JB, Kim H. Can a biomarker-based scoring system predict pathologic complete response after preoperative chemoradiotherapy for rectal cancer? *Dis Colon Rectum* 2014;57(5):592–601.
24. Yang C, Zhang J, Ding M, Xu K, Li L, Mao L, Zheng J. Ki67 targeted strategies for cancer therapy. *Clin Transl Oncol.* 2018;20(5):570–575.
25. Grem JL, Danenberg KD, Behan K, Parr A, Young L, Danenberg PV, Nguyen D, Drake J, Monks A, Allegra CJ. Thymidine kinase, thymidylate synthase, and dihydropyrimidine dehydrogenase profiles of cell lines of the National Cancer Institute's anticancer drug screen. *Clin Cancer Res.* 2001;7(4):999–1009.
26. Furusato B, Mohamed A, Uhlén M, Rhim JS. CXCR4 and cancer. *Pathol Int.* 2010;60(7):497–505.
27. González I, Bauer PS, Chapman WC, Alipour Z, Rais R, Liu J, Chatterjee D. Clinicopathologic determinants of pathologic treatment response in neoadjuvant treated rectal adenocarcinoma. *Ann Diagn Pathol.* 2020;45:151452.
28. Brown JR, DuBois RN. COX-2: A molecular target for colorectal cancer prevention. *J Clin Oncol.* 2005;23(12):2840–2855.
29. Edden Y, Wexner SD, Berho M. The use of molecular markers as a method to predict the response to neoadjuvant therapy for advanced stage rectal adenocarcinoma. *Colorectal Dis.* 2012;14(5):555–561.
30. Min BS, Choi YJ, Pyo HR, Kim H, Seong J, Chung HC, Rha SY, Kim NK. Cyclooxygenase-2 expression in pre-treatment biopsy as a predictor of tumor responses after preoperative chemoradiation in rectal cancer. *Arch Surg.* 2008;143(11):1091–1097.
31. Smith FM, Reynolds JV, Kay EW, Crotty P, Murphy JO, Hollywood D, Gaffney EF, Stephens RB, Kennedy MJ. COX-2 overexpression in pretreatment biopsies predicts response of rectal cancers to neoadjuvant radiochemotherapy. *Int J Radiat Oncol Biol Phys.* 2006;64(2):466–472.
32. Pauzas H, Gyvyte U, Latkauskas T, Kairevici L, Lizdenis P, Svagzdys S, Birgiolaite E, Kuliaviene I, Kupcinskas J, Tamelis A. The role of VEGFA, COX2, HUR and CUGBP2 in predicting the response to neoadjuvant therapy in rectal cancer patients. *Medicina* 2020;56(4):192.
33. Giralt J, Navalpotro B, Hermsilla E, de Torres I, Espin E, Reyes V, Cerezo L, de las Heras M, Ramon y Cajal S, Armengol M, Benavente S. Prognostic significance of vascular endothelial growth factor and cyclooxygenase-2 in patients with rectal cancer treated with preoperative radiotherapy. *Oncology* 2006;71(5–6):312–319.
34. Ogino S, Kirkner GJ, Noshio K, Irahara N, Kure S, Shima K, Hazra A, Chan AT, Dehari R, Giovannucci EL, Fuchs CS. Cyclooxygenase-2 expression is an independent predictor of poor prognosis in colon cancer. *Clin Cancer Res.* 2008;14(24):8221–8227.
35. Zhang X, Ma N, Yao W, Li S, Ren Z. RAD51 is a potential marker for prognosis and regulates cell proliferation in pancreatic cancer. *Cancer Cell Int.* 2019;19(1):356.
36. Vispe S, Cazaux C, Lesca C, Defais M. Overexpression of Rad51 protein stimulates homologous recombination and increases resistance of mammalian cells to ionizing radiation. *Nucleic Acids Res.* 1998;26(12):2859–2864.
37. Havelund BM, Sørensen FB, Lindebjerg J, Spindler K-LG, Jakobsen A. Pretreatment HIF-1 α and GLUT-1 expressions do not correlate with outcome after preoperative chemoradiotherapy in rectal cancer. *Anticancer Res.* 2011;31(5):1559–1565.
38. Shiota M, Takahashi T, Ishikawa H, Sakurai H, Ebara T, Suzuki Y, Saitoh J, Ohno T, Asao T, Kuwano H, Nakano T. Expression of hypoxia-inducible factor 1 α predicts clinical outcome after preoperative hyperthermo-chemoradiotherapy for locally advanced rectal cancer. *J Radiat Res.* 2011;52(6):821–827.
39. Kaemmerer D, Träger T, Hoffmeister M, Sipos B, Hommann M, Sängers J, Schulz S, Lupp A. Inverse expression of somatostatin and CXCR4 chemokine receptors in gastroenteropancreatic neuroendocrine neoplasms of different malignancy. *Oncotarget* 2015;6(29):27566–27579.
40. Zhang J, Liu C, Mo X, Shi H, Li S. Mechanisms by which CXCR4/CXCL12 cause metastatic behavior in pancreatic cancer. *Oncol Lett.* 2018;15(2):1771–1776.
41. Mai R, Kaemmerer D, Träger T, Neubauer E, Sängers J, Baum RP, Schulz S, Lupp A. Different somatostatin and CXCR4 chemokine receptor expression in gastroenteropancreatic neuroendocrine neoplasms depending on their origin. *Sci Rep.* 2019;9(1):4339.