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

Chiara Dalle Fratte,* Silvia Mezzalira,* 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, HIF1α, 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 HIF1α 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, HIF1α, 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 response[5].

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 life[6]. 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 stratification[7].

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 practice[8].

In the present study, a panel of tumor markers (MLH1, GLUT1, Ki-67, CA-IX, CXCR4, COX2, CXCL12, HIF1α, 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 repair[13], and the inflammation process[14]. 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 μm thick were stained with hematoxylin and eosin to be reviewed by a trained pathologist (V.C.). For immunohistochemistry, 3-μm-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, HIF1α (monoclonal, clone H1alpha67; 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

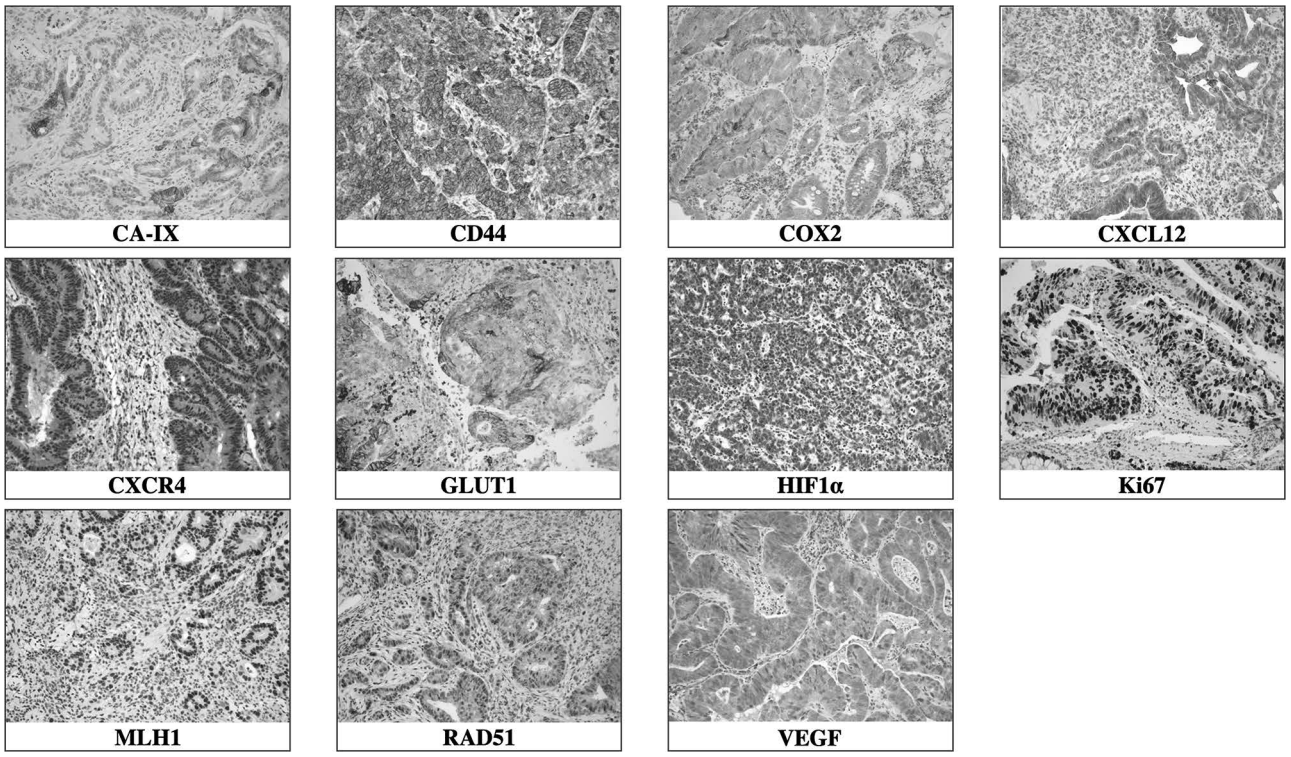

Figure 1. Immunohistochemical reactivity in pretreatment biopsies showing cytoplasmic, nuclear, and membrane reactivity for all the protein biomarkers investigated.

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 cytoplasmatic level for GLUT1, COX2, CXCL12, HIF1α, and VEGF; and at the nuclear and cytoplasmatic 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 method[15]. 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, HIF1α, 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	*p* Value*	TRG1	TRG2–4	*p* value*	TRG1	TRG2–4	*p* Value†
MLH1	25	62	6 (1–12)	8 (4–12)	$p = 0.126$	60 (20–90)	80 (40–90)	$p = 0.221$	64.0%	77.4%	$p = 0.430$
GLUT 1	25	70	6 (4–8)	6 (3–9)	$p = 0.903$	50 (30–60)	60 (40–70)	$p = 0.331$	84.0%	82.9%	$p = 0.864$
Ki-67	25	70	6 (3–9)	9 (6–9)	$p = 0.059$	40 (20–70)	60 (40–70)	$p = 0.070$	100%	100%	$p = 1.000$
CA IX	25	70	2 (1–2)	2 (0–2)	$p = 0.555$	5 (2–20)	10 (0–20)	$p = 0.721$	52.0%	48.6%	$p = 0.786$
CXCR4	20	61	3 (2–5)	2 (1–3)	$p = 0.010$	35 (20–50)	20 (10–50)	$p = 0.188$	59.1%	24.6%	$p = 0.002$
COX2	25	70	6 (3–8)	4 (3–6)	$p = 0.030$	70 (60–80)	70 (50–80)	$p = 0.649$	68.0%	41.4%	$p = 0.042$
CXCL12	25	70	2 (2–6)	3 (1–6)	$p = 0.778$	30 (20–50)	30 (10–50)	$p = 0.861$	64.0%	55.7%	$p = 0.067$
HIF1α	25	70	4 (2–6)	6 (2–8)	$p = 0.345$	70 (40–80)	70 (40–80)	$p = 0.910$	36.0%	55.7%	$p = 0.122$
VEGF	25	70	2 (1–3)	3 (1–6)	$p = 0.179$	40 (10–60)	60 (20–80)	$p = 0.081$	16.0%	28.6%	$p = 0.478$
CD44	25	70	6 (6–8)	6 (4–8)	$p = 0.608$	60 (50–80)	70 (40–70)	$p = 0.799$	92.0%	84.3%	$p = 0.848$
RAD51	25	68	4 (1–6)	4 (2–6)	$p = 0.254$	45 (20–50)	50 (30–65)	$p = 0.177$	59.1%	74.6%	$p = 0.350$

*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, HIF1α 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, HIF1α, 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		
	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 regression[10]. However, other studies showed that a higher rate of Ki-67-positive cells in treatment-naive 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 marker[24], 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 effect[26], 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 response[27]. 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 development[28], 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 investigators[34], we classified tumors as COX2 overexpressing when the immunostaining intensity was defined by the pathologists as "strong" to "moderate." However, when

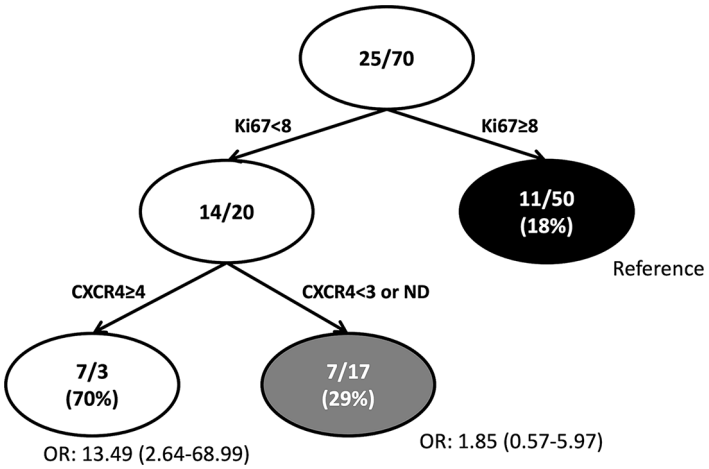

Figure 2. Classification and regression tree representation of the biomarkers' expression combination significantly predictive of pathological complete response (TRG1) in locally advanced rectal cancer patients. Fractions indicate the number of patients reporting a pathological complete response versus 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, HIF1α 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 HIF1α protein level[35]. Consistently with the biological connection between RAD51 and HIF1α, 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 radiotherapy[36]. HIF1α plays a key role in the cellular adaptation to hypoxia. A few studies reported conflicting findings upon the predictive role of HIF1α 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 HIF1α expression, measured by means of a semiquantitative score, has no predictive impact on the response to chemoradiotherapy[37]. Similarly, Shioya et al., who quantified the percentage of HIF1α-positive cells in 50 locally advanced rectal cancer patients, did not find significant associations with the pathological grading or pathological complete response[38]. 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 HIF1α 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 tumor[41], 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, HIF1α, 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.

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