Potential immune priming of the tumor microenvironment with FOLFOX chemotherapy in locally advanced rectal cancer

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ABSTRACT

Strategies to enhance tumor immunogenicity may expand the role of immunotherapy beyond the mismatch repair-deficient subtype. In this pilot study, biopsies were performed at baseline and after four cycles of FOLFOX in eight patients receiving neoadjuvant chemotherapy for stage II/III locally advanced rectal cancer. Immunostaining was performed for T cell subsets (CD3+, CD8+, CD45RO+); macrophages (CD163+); T regulatory cells (FOXP3+); and expression of MHC class I, PD-1 and PD-L1. Changes in cell number or intensity were quantified and correlated with treatment response. Pretreatment patterns of immune infiltrates were mixed and did not correlate with treatment response. Posttreatment increases in T cell infiltrates (CD3+, CD8+ and CD45RO+) and MHC-I expression were observed in five patients. CD163+ cell numbers increased in four patients. FOXP3+ cells numbers increased in two patients, decreased in two other patients and remained unchanged in three patients. PD-1 scores increased in seven patients, and PD-L1 scores increased in four patients. Changes in tumor T cell responses did not correlate with treatment response. Changes in FOXP3+ cells were associated with treatment response in some patients: two patients with increases in FOXP3+ cells had poor responses, whereas the patient with the greatest reduction in FOXP3+ cells had a complete response. The patient with a complete clinical response had a much higher increase in MHC-I expression than other patients. These results suggest that chemotherapy can increase immune activity in the tumor microenvironment and could potentially be utilized to prime immune responses prior to immunomodulatory treatments.

KEYWORDS: Locally advanced rectal cancer; Neoadjuvant therapy; Chemotherapy; Immune priming; Immunotherapy; T cells

Introduction

Immunotherapies have become routine in treatment of various cancers, such as melanoma, where

checkpoint blockade of CTLA-4 and PD-1 T-cell inhibitory molecules helps lengthen progression-free

survival and overall survival.[1] T-cell regulatory (Treg) receptor PD-1 and its ligand PD-L1 are

expressed in most cancers,[2] and together they can dampen the antitumor immune response.

Tumors thought to be susceptible to anti-PD-1 include those with high mutational load and

neoantigen burden [3] or with evidence of an immune-active tumor microenvironment (CD3/CD8+

infiltrates and MHC class I expression).[4,5] Intratumoral PD-1/PD-L1 expression and presence of T

cells within the tumor microenvironment are thought to represent the most useful indicator that

anti- PD-1 therapy may be effective.

To date, the role of immunotherapy in colorectal cancer (CRC) has been limited to the mismatch

repair-deficient (dMMR) subtype. dMMR tumors are strongly immunogenic, with high numbers of

tumor-infiltrating lymphocytes thought to be responding to neoantigens brought about by the high

mutational burden.[6] Compared with mismatch repair-proficient (pMMR) CRC, dMMR tumors also

express higher levels PD-1/PD-L1.[7,8] Phase II trials have shown that anti-PD-1/PD-L1 agents help

lengthen progression-free and overall survival in patients with stage IV dMMR CRC.[9,10] However,

since dMMR tumors account for only 25%, 12% and 4% of stage II, III and IV CRCs, respectively,

immunotherapy is a treatment option in only a small subset of CRCs.

The majority of pMMR CRCs exhibit low numbers of T cells in the tumor microenvironment, along

with low levels of PD-1/PD-L1 expression; therefore, checkpoint blockade immunotherapy is not

thought to be effective.[11] Strategies aimed at enhancing immunogenicity in pMMR CRC may allow

broader use of immunotherapy. Along this line of reasoning, conventional cytotoxic chemotherapy

and radiotherapy (RT) are thought to result in immunogenic cell death, with T cell infiltrates

responding to release of antigen from damaged cells.[12] Such treatments may synergize with

immunotherapies, enhancing treatment response. Trials are currently under way employing

combinations of immunotherapies with immune-priming strategies in gastroesophageal, head and

neck, and lung cancers (NCT02872116, NCT02764593 and NCT02944396 on

www.ClinicalTrials.gov).[13]

Observational data suggest that RT enhances T cell infiltration in locally advanced rectal cancer, and

preclinical models suggest that cytotoxic chemotherapy may have similar effects.[14-18] However, the

effect of cytotoxic chemotherapy on intratumoral immune responses in patients with CRC has not

previously been studied. In this prospective pilot study, we investigated whether conventional

cytotoxic chemotherapy can induce immune priming in pMMR locally advanced rectal cancer.

Results

Matched biopsies taken before and after four cycles of induction FOLFOX chemotherapy (folinic acid,

fluorouracil and oxaliplatin) were collected in eight patients: six men and two women, ages 33-78

years. The clinical and pathological characteristics of the cohort are listed in **Table 1**. All eight

patients had clinical stage III (node-positive) disease; two had cT4 tumors, five had cT3 tumors, and

one had a cT2 tumor. The distance of the tumor from the anal verge was $\leq$5 cm in three patients, 5-

10 cm in three other patients and >10 cm in two patients.

Pretreatment MMR status was available for seven patients; all seven had retained expression of

MMR proteins. The number of days between the index biopsy and completion of four cycles of

FOLFOX ranged from 61 to 127 days (mean 82 days). By the completion of neoadjuvant treatment,

six patients had downstaging of T stage and all eight patients had downstaging of N stage. Two

patients had a poor clinical response, five patients had an incomplete response, and one patient had a complete response, based on digital exam, endoscopy and MRI.

The data on pre- and posttreatment tumor immune responses are shown in **Table 2** and **Figure 1**. Pretreatment patterns of immune infiltrates were mixed and did not appear to correlate with responses to treatment. CD3+ and CD8+ infiltrates increased in five patients, CD45RO+ infiltrates increased in four patients, and CD163+ cells increased in four patients. The MHC-I histoscore increased in five patients, the PD-1 score increased in seven patients, and the PD-L1 score increased in four patients. FOXP3+ infiltrates increased in two patients, decreased in two other patients and remained unchanged in three patients (pretreatment FOXP3+ data was not available for one of the eight patients). The two patients with increases in FOXP3+ cells had poor responses to treatment, whereas the patient with the greatest reduction in FOXP3+ cells had a complete clinical response (**Fig. 2**). The patient with a complete response had a much higher increase in MHC-I expression within the tumor than the other patients.

Discussion

The findings of our pilot study suggest that FOLFOX chemotherapy is capable of inducing changes in the immune contexture within the tumor microenvironment, such as increases in T cells (CD3+, CD8+ and CD45RO+) and in MHC-I and PD-1 expression. These changes are similar to those observed in preclinical models after chemoradiotherapy (CRT) secondary to immunogenic cell death with antigen release, increased MHC-I and cross-presentation to T cells.[15-19] In rectal cancer patients treated with CRT, higher numbers of CD4+/CD8+ cells in resected specimens than in pretreatment biopsies have been reported.[14,20] CRT also induces systemic immune changes, including reductions in circulating Tregs and myeloid-derived suppressor cells.[21] This immunomodulation may account for the reported abscopal effects on distant metastases after RT to the primary tumor, potentially mediated by cross-priming of T cells following tumor cell death.[12, 18,19]

Our results are consistent with previous reports of association between changes in Tregs and treatment response. We observed that tumors in which FOXP3+ Tregs increased had a poor treatment response. The role of Tregs in CRC is not clear, but they are known to harbor immunosuppressive activity, dampening T cell responses.[22] Low numbers of FOXP3+ Tregs in posttreatment resection specimens have been reported to be associated with greater treatment response after CRT.[23] No strong relationship between pretreatment Tregs and treatment response has been observed, suggesting that the type of immune response generated by RT is more important.[24]

The patient with a complete clinical response had the largest increase in MHC-I expression. High MHC-I expression reflects increased antigen presentation and is considered a measure of an immune-active microenvironment; it may therefore indicate ongoing immunogenic cell death in response to FOLFOX. Further work is required to test this hypothesis and to determine whether MHC-I has potential as an early biomarker of response during chemotherapy treatment.

We found no appreciable relationship between T cell changes and treatment response, even for tumors with a 200% increase in T cell numbers. Further work is required to determine whether the effects of these T cell responses are mitigated by the presence of inhibitory immune checkpoints (e.g., PD-1 and CTLA-4) or other T cell suppressors (myeloid-derived suppressor cells or Tregs).

No biomarkers of response to immunotherapies (e.g., anti-CTLA-4 and anti-PD-1/ PDL1) are available, but pretreatment evidence of an immune-active tumor microenvironment (e.g., high T cells and MHC-I expression) is considered important.[3-5] Where such features are absent, strategies to prime the microenvironment in order to induce immune responses are sought. FOLFOX may be capable of achieving these desirable effects in a proportion of patients. Sequencing of

chemotherapy and RT to optimize immunological sequelae is likely to be critical to successful

utilization of immunotherapy and requires further investigation. The relative ease with which tumor

biopsies were collected from outpatients in our study indicates that rectal tumors are ideal for

studying the scheduling of priming treatments.

In summary, the results of our pilot study indicate that FOLFOX chemotherapy in patients with

locally advanced rectal cancer was associated with increases in T cell infiltrates, MHC-I expression

and PD-1 expression. It is likely that the immunogenicity generated in response to chemotherapy

plays a role in treatment response, and evidence of this role may be more apparent in a larger study.

Our findings suggest that neoadjuvant chemotherapy can potentially be utilized to prime immune

responses prior to immunomodulatory treatments. These observations provide support for ongoing

work investigating the role of immune stimulation by conventional treatments in rectal cancer and

for future trials aimed at evaluating combinations of chemotherapy, RT, and immunotherapy.

Patients and Methods

Patients

The study population consisted of nonconsecutive patients seen over a 4-month period with locally

advanced rectal cancer clinically staged cT3/4 or N+ (American Joint Committee on Cancer stage II

and III) who received neoadjuvant therapy prior to planned rectal resection. As per National

Comprehensive Cancer Network guidelines, the patients received eight cycles of induction FOLFOX

prior to CRT.[25] With approval from the institutional review board, we prospectively evaluated the

immune contexture of tumor biopsies taken at baseline and after four cycles of FOLFOX

chemotherapy. All patients were considered to have pMMR tumors based on pretreatment biopsy

immunohistochemistry (no loss of MLH1, PMS2, MSH2 or MSH6 protein expression).

Samples

Immunohistochemistry was performed on matched samples (biopsy at initial diagnosis and after four cycles of FOLFOX) to grade infiltrates of T cells (CD3+, CD8+, CD45RO+), Tregs (FOXP3+) and macrophages (CD163+) (**Fig. 3**). We also evaluated MHC-I (as a measure of antigen presentation) and PD-1/PDL-1 expression. Sections of 4-μm thickness were obtained from paraffin-embedded biopsy samples. After deparaffinization and rehydration, sections were incubated with primary monoclonal antibodies recognizing CD3 (LN10, 1:100; Leica Biosystems), CD8 (SP57, ready to use; Ventana), CD45RO (UCHL1, 1:200; Dako), FOXP3 (236A/E7, 1:500; Abcam), CD163 (MRQ-26, ready to use; Cell Marque), MHC-I (A4; Thermo Fisher Scientific), PD-1 (NAT105, ready to use; Cell Marque), or PD-L1 (E1L3N, 1:250; Cell Signaling Technology). We used the staining platforms BenchMark Ultra from Roche (CD3 and CD163) and BenchMark Ultra from Ventana (CD8) and the detection systems OptiView from Ventana (CD3, CD8, CD163 and PD-1) and Bond Polymer Refine from Leica Biosystems (CD45RO, FOXP3, MHC-I and PD-L1).

Immune response

Immune cells expressing CD3+, CD8+, CD45RO+, FOXP3+ or CD163+ were quantified in biopsy specimens using a cell counting approach. For each specimen, three high-power fields containing both tumor stroma and epithelium were selected at random. Cell counts were averaged over the three fields. MHC-I expression was assigned a modified histoscore obtained by multiplying the percentage of cells expressing MHC-I by the intensity of staining, which was graded on a scale of 0 to 3. Histoscore values therefore ranged from 0 to 300. PD-1 and PD-L1 were assessed using a previously described method.[26] Briefly, tumor cells staining for PD-L1 and tumor-infiltrating lymphocytes expressing PD-1 were graded on a scale from 0 to 2 (0, no staining; 1+, faint or weak staining; 2+, moderate or strong staining). Increases or decreases of >25% in absolute cell counts or antibody staining intensity, in addition to any point change in the PD-1/PD-L1 scores (0-2) in posttreatment samples, were categorized as changes in the immune response. C.R. scored all the slides, with dual scoring for three patients by J.S. to ensure consistency.

Staging and clinical response

Pretreatment staging was based on MRI data. Posttreatment staging was based on MRI/CT data in patients with a complete clinical response or on resection specimen pathology in patients undergoing surgery. A downward posttreatment change in T or N stage (e.g., T4 to T3 or N1 to N0) was categorized as downstaging. Complete clinical response was defined as absence of clinical, endoscopic or radiographic evidence of tumor at completion of neoadjuvant therapy. At Memorial Sloan Kettering, such patients are offered the option of nonoperative, watch-and-wait management. A reduction of <50% in tumor bulk was categorized as poor response, and a reduction of 50-99% was categorized as a good but incomplete response.

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Figure 1. Tumor microenvironment changes in counts of immune cells and expression of MHC-1, PD-1 and PDL-1 that occurred during FOLFOX treatment. Complete, good, and incomplete responses to treatment are indicated by light grey, dark grey, and black lines, respectively. hpf, high-power field.

Figure 2. Immune responses in relation to treatment responses. The PD-1 score did not change for one patient with a good response; the PD-L1 score did not change for one patient with a poor response.

Figure 3. Representative micrographs of immunostaining for antibody expression in rectal cancer biopsies. Scale bars: 500 μm for FOXP3+, 1 mm for all others.

Table 1. Clinical and pathological characteristics of the study cohort.

Patient	Age (yr)	Sex	Initial MRI Stage	Distance from Anal Verge (cm)	Days between Biopsies[a]	Posttreatment Stage	Downstaging		Complete Response
							T	N	
1	63	M	cT4b N1	7.6	82	ypT1 N0	Yes	Yes	No
2	69	M	cT3c N1	5.0	62	ypT2 N0	Yes	Yes	No
3	67	F	cT4b N1	3.2	81	ypT2 N0	Yes	Yes	No
4	75	M	cT2 N1	6.5	89	ycT0 N0	Yes	Yes	Yes
5	34	M	cT3c N2	13.0	78	ypT3 N1	No	Yes	No
6	78	M	cT3b N1	2.8	127	ypT2 N0	Yes	Yes	No
7	48	F	cT3b N2	5.8	61	ypT3 N1	No	Yes	No
8	33	M	cT3b N1	12.0	74	ypT2 N0	Yes	Yes	No

[a]All patients had received four cycles of FOLFOX by the time of the second biopsy.

Table 2. Pre- and posttreatment immune responses.[a]

Biopsy	Cells/HPF					MHC-I Histoscore	PD-1 Score	PD-L1 Score
	CD3+	CD8+	CD45RO+	FOXP3+	CD163+			
Patient 1								
Pre	23	22	20	10	22	220	1	2+
Post	*5*	*5*	*7*	*6*	*10*	**280**	**2+**	*1+*
Patient 2								
Pre	13	6	17	8	10	175	1+	0
Post	**21**	**18**	**29**	7	**16**	**270**	1+	**1+**
Patient 3								
Pre	14	12	22	10	10	200	0	1+
Post	**19**	**17**	26	12	**15**	230	**1+**	*0*
Patient 4								
Pre	15	8	16	17	15	45	0	0
Post	**25**	**14**	**23**	*5*	14	**210**	**1+**	**1+**
Patient 5								
Pre	9	3	15	5	11	180	0	0
Post	**14**	**9**	**22**	**8**	**16**	**230**	**1+**	**1+**
Patient 6								
Pre	21	15	22	NA	15	210	1+	1+
Post	22	*7*	23	5	14	*125*	**2+**	*0*
Patient 7								
Pre	20	8	15	8	12	220	0	0
Post	*10*	9	**20**	**14**	14	210	**1+**	0
Patient 8								
Pre	8	5	17	5	9	155	0	0
Post	**22**	**12**	15	4	**14**	**405**	**1+**	**1+**

[a]Increases of >25% are boldfaced. Decreases of >25% are italicized. HPF, high-power field; NA, not available.