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Research Article

The Predictive Role of Immunohistochemical FoxP3 and CD163 Expression in Response to Neoadjuvant Therapy of Rectal Carcinomas

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Abstract

Objectives: In the present study, we aim to investigate the predictive role of FoxP3 and CD163 expressions in biopsy samples for the neoadjuvant treatment, which are performed pretreatment due to locally advanced rectum cancer.

Methods: The study included 70 patients who were operated post-neoadjuvant 5FU-based chemoradiotherapy (nCRT) in 2011-2019 due to locally advanced rectum cancer.

Results: Forty-eight (68.6%) of the patients involved in our study were men and 22 (31.4%) were women. The median age was 64.81±10.16 years (37-84 years). For FoxP3, considering all the tumor areas in the biopsy material, the average number of FoxP3-positive lymphocytes was 12.37±11.68/1 HPF (median: 9.8; min: 0.2, max: 66). The number of CD163-positive macrophages was low in 30 (42.9%) tumors, and high in 40 (57.1%). As a result of regression evaluations, 16 (22.9%) cases were evaluated in Tumor Regression Grade (TRG) 1 category, 23 cases in TRG2, 12 cases in TRG3, 14 cases in TRG4 and 5 cases in TRG5 category. There was a significant relationship between the FoxP3 increase and the tumor regression grade, with no significant relationship between the intensity of CD 163 and the tumor regression grade. In addition, a significant relationship was found between the regression grade and tumor differentiation, perineurial invasion, positivity of surgical margin, residual tumor depth and lymphatic response.

Conclusion: Our study supports that the T-cell-mediated immune response plays an important role in the tumor response to nCRT, and particularly the FOXP3+ TIL densities are associated with the pathological response to nCRT.

Keywords: Rectal cancer, Fox P3, CD 163, Tumor regression Grade

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Colorectal cancer remains one of the leading causes of worldwide deaths by cancer.[1] For patients with locally advanced rectum cancer (stage II and III), neoadjuvant chemoradiotherapy (nCRT) can significantly reduce the toxicity associated with local recurrence and treatment, and more importantly, make tumors more viable for resection.[2] Approximately 15% to 30% of the patients can present a pathological complete response.[3,4] A better understanding and determination of predictive factors in the neoadjuvant treatment response raises the question of preferring risk-based treatment approaches, such as using more aggressive preoperative treatment regimens

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for patients with less chance of responding. On the contrary, less radical strategies such as local incision, even non-surgical follow-up, can be preferred in cases where the likelihood of good pathological response can be determined preoperatively with accuracy.[5,6] To enhance the prediction of sensitivity to therapies, recent years have witnessed the development of novel methods, including novel biomarkers proposed.[7] It has been reported that inflammatory cell infiltration in patients with colorectal cancer is associated with survival rates regardless of the pathological stage.[8] Tumor-infiltrated lymphocytes (TIL), an important component of the tumor microenvironment, are defined in many organ cancers and considered to be an indicator of host immune response to the tumor cell. In general, M1 macrophages, NK, CD8+T lymphocytes, Th1 cells are associated with tumor suppression, while M2 macrophages, Treg, Th2 cells are considered associated with tumor progression.[9] Treg cells express the biomarkers CD4, FOXP3, and CD25 and are thought to be derived from the same lineage as naïve CD4+ cells.[10] One of the most commonly used markers for self-tolerance promoting regulatory T-cells (Treg) is the transcription factor forkhead box protein P3 (FoxP3).[11] High levels of Treg cells around the tumor microenvironment are associated with poor prognosis in many cancers, such as ovarian, breast, kidney, and pancreatic cancer.[12,13] This indicates that Treg cells suppress the effector T-cells and block the body's immune response to cancer. However, in some types of cancer, the opposite is true and high levels of Treg cells are associated with a positive prognosis. This trend is seen in cancers such as colorectal carcinoma and follicular lymphoma. This is thought to be due to the ability of Treg cells to suppress common inflammation that is known to trigger cell proliferation and metastasis.[12]

One of the most common types of cells in the tumor microenvironment is Macrophages, which play a central role in inflammation and tumor development. Macrophages, among the stromal cell groups in the tumor microenvironment, are increasingly becoming a focus of interest due to their function in the progression of tumor neovascularization and their importance in the metastatic process.[14] CD68 is used as the surface antigenic determinant that determines M1 macrophages, and CD163 and CD206 are used as the surface antigenic determinants that determine M2 macrophages.[15] It remains unknown whether the FoxP3+ lymphocytes and CD163+ macrophages, which are shown to be effective in many tumors, are useful in predicting neoadjuvant treatment response in colorectal cancers. Based on the hypothesis that it can be used as a target molecule in determining possible treatment protocols and personalized treatment, this study aims to investigate the predictive importance of immunohistochemical FoxP3 and CD163 expressions in neoadjuvant treatment in the context of colorectal cancers.

This study has been approved by the institutional review board of our university (Date: 12/05/2020, No. 174), and all patients provided written informed consent.

Methods

Clinicopathological data

Patients diagnosed with rectal adenocarcinoma by biopsy between 2011 and 2020 in a single center were scanned from the electronic archive. Seventy patients who completed neoadjuvant treatment with some regimen and were operated afterwards were included in the study. Patients who did not undergo surgery in our hospital, could not complete the neoadjuvant treatment, did not have sufficient tumor tissue in the biopsy material, and whose biopsy and/or surgical materials were not suitable for histopathological and immunohistochemical examination, and tumors that did not have adenocarcinoma morphology were excluded from the study.

All sections taken from the diagnostic biopsy materials embedded in paraffin blocks and stained with Hematoxylin & Eosin (H&E) after the routine tissue follow-up of the patients were re-examined. The degree of histological differentiation in tumors was evaluated and classified as good-moderate-poor. For lymphocytic infiltration, a 4-point scoring system was used as Zhang et al.[16] According to this system; score 0 refers to no infiltrating lymphocytes; score 1 to a mild increase of infiltrating lymphocytes in the tumor nest or stroma; score 2 to increased infiltrating lymphocytes interwoven with tumor tissue; and score 3 to prominent infiltrating lymphocytes separating or incorporated in tumor tissue.

The entire ulcerated area/tumor/lesion observed in the operation materials of the patients was subjected to the histopathological examination. Sections obtained from tissues embedded in paraffin blocks after routine tissue follow-up were stained with H&E and examined under a light microscope. Histopathological criteria in each case were evaluated according to the 2018 edition of the World Health Organization Gastrointestinal System Tumors. Primary tumor (ypT) was classified as ypT0, ypT1, ypT2, ypT3, ypT4, and lymph node metastasis (ypN) was classified as ypN0, ypN1, ypN2. The presence of macroscopic and/or microscopic tumor at the surgical margin was accepted as positive. Perineural invasion (PNI) and lymphovascular invasion (LVI) were classified as present or absent. The tumor regression grade of the resected tumor was assessed using

the original score proposed by Mandard et al.[17] Tumor regression was described as follows: TRG1: no viable cancer cells, complete response; TRG2: single cells or small groups of cancer cells; TRG3: residual cancer outgrown by fibrosis; TRG4: significant fibrosis outgrown by cancer; TRG5: No fibrosis with extensive residual cancer. While evaluating the sections of the operation material, the pathologists were blind to the clinical information of the patients and the histopathological features of the biopsy material taken before the treatment.

The electronic archive system of our hospital was used for the demographic data of the patients and the tumor size observed in the operation material.

Immunohistochemistry

For immunohistochemical (IHC) investigation, 4-micron-thick sections were obtained from biopsy material block that had gone through the routine process. Immunohistochemically, FoxP3 (Abcam, Clone:EP340, 1/100dilution, catalog number: AC-0304RUO) and CD163 (Biocare Medical, Clone: 10D6, 1/100 dilution, catalog number: CM353AK) primary antibodies were used. Staining was performed according to standard protocols provided by the automated Ventana BenchMark XT immunostainer (Ventana Medical Systems, Inc., Tucson, AZ, USA). The positive control was tonsil tissue for FoxP3 and CD163. Each sample was evaluated by at least one pathologist without information of the corresponding data (Fig. 1).

For FoxP3, all tumoral areas in a section of biopsy material were examined with a x100 (HPF) objective. FoxP3 positive lymphocytes were counted, and the number obtained was divided by the number of HPF examined to calculate and record the mean number of FoxP3 positive lymphocytes in 1 BBA (FoxP3+cells/1 HPF).

For CD163, all tumoral areas in a section of biopsy material were examined with a x100 (HPF) objective. High infiltration of CD163+ macrophages was defined as more than average 100 positive cells/1 HPF, as described by Pan et al.[18] CD163+ macrophage count below this value was considered low infiltration.

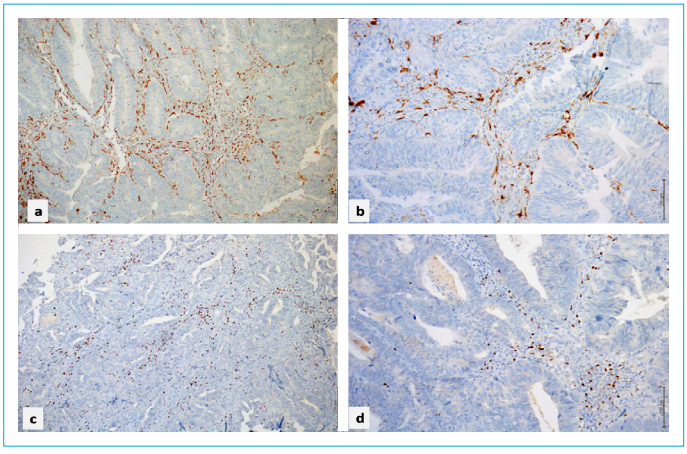

Figure 1. Characteristic microscopic appearance of CD163 and Fox P3. **(a)** Microscopic view from the case with high CD163 (immunohistochemistry, anti CD163, x10). **(b)** Microscopic view from the case with low CD163 (immunohistochemistry, anti CD163, x20). **(c)** Microscopic appearance of tumor with extensive FoxP3+ cell infiltration (immunohistochemistry, anti FoxP3, x4). **(d)** Microscopic appearance of the tumor with a small number of FoxP3+ cell infiltration (immunohistochemistry, anti FoxP3, x20).

Statistical Analysis

The statistical analyses were performed with the SPSS software version 21.0. "Cox regression test", and "Pearson's Chi-Square test" were used. A p-value ≤ 0.05 was considered statistically significant in all statistical analyses.

This study followed the Declaration of Helsinki on medical protocol and ethics, and the regional Ethical Review Board approved the study.

Results

There were 48 men (68.6%) and 22 women (31.4%). The median age was 64.81±10.16 years (range, 37-84 years).

Histopathological Features of Biopsy Materials

When the histological sections of the biopsy materials were re-evaluated, 10 (14.3%) of the tumors were well differentiated, 55 (78.6%) moderately differentiated, and 5 (7.1%) poorly differentiated adenocarcinomas. Although a four-grade rating system was used for lymphocytic response, grade 3 lymphocytic response was not observed in any of the cases. There were 33 (47.1%) tumors without a lymphocytic response, 15 (21.4%) tumors with a score 1 lymphocytic response, and 22 (31.4%) tumors with a score 2 lymphocytic response. Demographic and histological features according to Mandard's regression scoring is shown in Table 1.

FoxP3-positive lymphocyte count/1HPF observed in diagnostic biopsies of regression score groups in the operation material are shown in Table 2.

The relationship between histological and demographic features and the degree of lymphocytic infiltration is shown in Table 3.

Immunohistochemistry

Considering all the cases, the mean FoxP3 positive lymphocyte count was 12.37±11.68 /1 HPF (median: 9.8; min:0.2, max:66). The number of CD163 positive macrophages was low in 30 (42.9%) of the tumors and high in 40 (57.1%).

Histopathological Features of Operation Materials

The mean size of tumor was 2.89±1.39cm (range, 0.30-

Table 1. Distribution of case numbers of demographic and histological features according to Mandard's regression scoring.

	TRG 1	TRG 2	TRG 3	TRG 4	TRG 5	p
Sex						
Female	7	5	5	4	1	0.546
Male	9	18	7	10	4	
Differentiation						
Well-differentiated	1	8	0	0	1	0.034
Moderately differentiated	13	14	11	14	3	
Poorly differentiated	2	1	1	0	1	
Lymphovascular invasion						
Absent	16	20	8	10	3	0.082
Present	0	3	4	4	2	
Perineural invasion						
Absent	16	20	9	12	2	0023
Present	0	3	3	2	3	
Surgical Margin						
Negative	16	23	9	13	3	0007
Positive	0	0	3	1	2	
Depth of Invasion						
ypT0	16	1	0	0	0	<0.0001
ypT1	0	2	0	0	0	
ypT2	0	6	4	5	1	
ypT3	0	13	6	9	3	
ypT4	0	1	2	0	1	
Lymph Node Metastasis						
ypN0	15	15	6	11	2	0136
ypN1	1	3	4	2	1	
ypN2	0	5	2	1	2	
Lymphocytic Response						
Score 0	11	14	4	3	1	0004
Score 1	1	5	6	3	0	
Score 2	4	4	2	8	4	
CD163 density						
Low	8	9	7	5	1	0559
High	8	14	5	9	4	

7.5cm). In the grading depending on the depth of invasion, 17 (24.3%) tumors were ypT0, 2 (2.9%) ypT1, 16 (22.9%) ypT2, 31 (44.3%) ypT3 and 4 (5.7%) were ypT4. No lymph node metastasis was observed in 49 (70.0%) cases, but 11 (15.7%) cases had lymph node metastasis in the ypN1 category and 10 cases in the ypN2 category. Peritumoral lymphovascular invasion was detected in 13 (18.6%) cases and perineural invasion was found in 11 (15.7%) cases. There were only 6 (8.6%) patients with tumor at the surgical margin.

Table 2. Data table showing FoxP3-positive lymphocyte count/1HPF observed in diagnostic biopsies of regression score groups in the operation material.

	Number of FoxP3 positive lymphocyte /1 HPF				
Regression score	Mean	Std Dev	Minimum	Maximum	p
TRG 1	14.75	12.12	2.00	52.60	0008
TRG 2	17.94	13.27	1.00	66.00	
TRG 3	9.00	5.99	0.20	20.00	
TRG 4	6.08	9.22	0.20	33.00	
TRG 5	4.80	3.81	0.20	10.60	

As a result of regression evaluations, 16 (22.9%) cases were in the TRG1 category, 23 cases were in the TRG2 category, 12 were in the TRG3 category, 14 were in the TRG4 category, and 5 were in the TRG5 category.

Discussion

Today, neoadjuvant treatment is considered a standard treatment approach for post-CRT total mesorectal excision (TME) stage II and III rectal cancer.[19,20] Treatment response rates show significant heterogeneity between patients,

Table 2. Relationship between histological and demographic features and the degree of lymphocytic infiltration.

	Lymphocytic infiltration			Total	p
	Score 0	Score 1	Score 2		
Gender					
Women	12	5	5	22	0.55
Men	21	10	17	48	
Differentiation					
Well	8	2	0	10	0.04
Moderate	21	13	21	55	
Poor	4	0	1	5	
Depth of Invasion					
ypT0	12	1	4	17	0.20
ypT1	2	0	0	2	
Ypt2	6	3	7	16	
Ypt3	12	9	10	31	
Ypt4	1	2	1	4	
Lymph Node Metastasis					
ypN0	23	8	18	49	0.39
ypN1	6	3	2	11	
ypN2	4	4	2	10	
LVI					
None	30	9	18	57	0038
Positive	3	6	4	13	
PNI					
None	29	11	19	59	0.41
Yes	4	4	3	11	
CD163					
Mild	14	10	6	30	0059
Medium	19	5	16	40	

Perineural invasion (PNI) and lymphovascular invasion (LVI) were classified.

which increases the need for predictive factors to guide the treatment decision. On the other hand, the factors that would predict the response to neoadjuvant treatment in rectal cancer are not clearly defined. In various studies, some clinical factors and molecular determinants such as tumor size, TNM stage, radiation and fractionation, and time between neoadjuvant chemoradiotherapy and surgery, carcinoembryonic antigen (CEA) level, epidermal growth factor receptor (EGFR) and P21 were identified as the predictor of the treatment response.[21,22] However, clinical and radiological parameters have so far only reached a limited specificity and sensitivity.[23] Recent studies show that the tumor microenvironment has a significant impact on cancer development and progression. Tumor-infiltrating lymphocytes (TILs) are an important component of this microenvironment and play a vital role in tumor progression and treatment response. The population of tumor-infiltrating T-cells mainly consists of CD8+ cytotoxic T-cells, CD4+ T-helper cells and CD4+ regulatory T-cells (Tregs). CD8+ T-cells are the key effector cell population that mediates effective anti-tumor immunity and improves clinical results Tregs are believed to protect the host and maintain systemic immune homeostasis to prevent autoimmune disease by suppressing self-reactive cells.[24,25]

The prognostic significance of FoxP3+ tumor-infiltrating Treg in colorectal cancer (CRC) and rectal cancer (RC) is a subject that has been studied frequently, although controversial. Some studies have reported that tissue infiltrating lymphocytes (TILs) are a prognostic factor for colorectal cancer and are associated with tumor regression grade (TRG) after nCRT.[26,27] However, even the basic question of whether or not increased Treg infiltration in CRC is a positive or negative prognostic factor remains unclear; and many studies have reported contradictory results.[28,29] In patients with rectal cancer, CD4+, CD8+ and PD-L1-positive TILs may change after nCRT and may serve as prognostic factors or histological predictors.[30,31] While Treg is generally assumed to be immunosuppressive and therefore to have a negative prognostic effect, there is ample evidence that the density of high intratumoral FoxP3+T-cells can be an indicator of improved prognosis in CRC and other types of cancer.[32-35] However, numerous studies associate increased T-cell densities of FoxP3+ with a negative prognosis, reporting otherwise.[36-38] Some of these inconsistencies are likely to be the result of differences in measurement and treatment methods. However, the apparent prognostic difference is an indication of the possibility that more than one subpopulation of FoxP3+ T-cells can be present in CRC or that Treg may have different and contrasting functions from time to time, depending on other environmental factors.[39] The relative location of the CD8+ and FoxP3+ T-cells and the distance from the tumor cells (stromal and intra-tumor) can also help explain some differences in correlation between patient survival and histochemical analyses of TILs in rectal cancers. In fact, a study that explored the distance between the FoxP3+ and CD8+ T-cells before and after chemoradiotherapy in the stroma and tumor reported that the short distance between the two types of cells in the tumor epithelium is associated with positive prognosis, and the opposite was observed in the stromal compartment.[40] A study comparing pre- and post-chemoradiotherapy biopsies from patients with rectal cancer showed that stromal CD8+T-cells increased while stromal FoxP3+TILs remained stable.[41] Post-treatment, high stromal CD8+ TIL numbers were found to be strongly related to better prognosis, and the pretreatment high intraepithelial ratio CD8/FoxP3 was found to be a sign of tumor regression. Neoadjuvant chemoradiotherapy has also been reported to increase the density of CD4+ TILs, but the

CTLA-4 exposure and FoxP3+ densities were maintained.[42] In an immunohistochemical analysis of surgical specimens obtained from LARC patients, it was reported that post-radiotherapy low stromal FoxP3+ cell density was associated with a favorable regression grade.[33] However, when biopsies from these patients were analyzed prior to treatment, neither FoxP3+ nor CD8+ T-cells showed correlation with the treatment outcome.[43] In addition, in another study, the high FoxP3+ TIL density after radiotherapy showed that it is related to better progression-free survival.[44] In the same study, there was no correlation between pre-treatment CD8+ TILs and survival, while the post-treatment reduction of CD8/FoxP3 ratio predicted better overall and progression-free survival. A more recent study comparing the biopsy material from rectal tumors pre-treatment and 7 days after radiotherapy showed that pre-treatment high-density CD4+ and FoxP3+ cells are significantly related to tumor shrinkage.[45] Miyakita H. et al. studied the changes in the density of tumor-infiltrating lymphocytes (TILs) before and after chemoradiotherapy and clinical benefits in patients with rectal cancer. In their study, patients presenting high-density FoxP3+T-cell in biopsy samples that were taken before and 7 days after nCRT started were associated with a good histological response. In our study, a significant relationship was found between the FoxP3 density in the pre-nCRT biopsy samples and the tumor regression degree. These results suggest that high-density TIL tumors are originally associated with a high immunogenic condition, promoting the release of tumor-specific antigens and causing a good response to the nCRT.[39]

The results of numerous studies have shown that the TAMs are associated with poor clinical course according to tumor type and localization.[15] TAMs are commonly defined by CD163 or CD206 surface markers. Immunohistochemical studies using various human tumor tissues show that a TAM number exceeding 80% is associated with poor clinical prognosis. A study has demonstrated that TAMs represent an independent prognostic factor in esophageal squamous cell carcinoma. In addition, high-density TAMs are associated with the aggressive properties of gastric cancer and appear as an independent prognostic indicator for patients with stomach cancer.[46] Tumor-associated macrophages (TAMs) have been shown to facilitate breast carcinogenesis and its increase is particularly correlated with worse clinical result and resistance to chemotherapy.[47] Molecule CD163 associated with macrophages has been reported to be a prognostic biomarker of different types of cancer, but its role in colorectal cancer (CRC) is unclear. Recent meta-analyses have shown that a high lymphocyte-monocyte ratio is a significant predictive for better overall survival (OS), disease-free survival (DFS) and cancer-specific survival in CRC patients, given the peripheral blood leukocytes.[48] However, the circulated monocyte subgroups and monocyte-macrophage marker CD163 have not been widely investigated for CRC patients. For example, a high CD163+ TAM density was reported to be related to both negative and positive clinical outcome in CRC.[49-56] A study by Krijgsman D. et al. researched CD163, which is expressed by the circulating monocytes and TAMs, and its circulating, soluble form (sCD163) in relation to the clinicopathological parameters in CRC. As a result, the sCD163 and monocytes in circulation may be potential prognostic biomarkers in CRC patients, while the TAMs in the tumor showed no relation to the clinical outcome. Our study found no relationship between the CD163 level and tumor regression.

This study had some limitations. First of all, the patients were all different in terms of standard chemoradiotherapy (CRT) dose and fraction, as well as the time until surgery. Also, there was no comparison of post-operational surgery materials with bx materials of the patients.

Conclusion

Our study supports that the T-cell-mediated immune response plays an important role in the tumor response to nCRT, and particularly the FOXP3+ TIL densities are associated with the pathological response to nCRT. Our results suggest that we have found a possible explanation for the inconsistent findings related to the role of Treg reported in previous studies, and we believe that the FoxP3 density in the preoperative biopsy material in the neoadjuvant treatment management of rectal cancer is particularly important.

Disclosures

Ethics Committee Approval: This study has been approved by the institutional review board of Izmir Bozyaka Training and Research Hospital (Date: 12/05/2020, No. 174).

Peer-review: Externally peer-reviewed.

Conflict of Interest: None declared.

Authorship Contributions: Concept – O.O., T.S., A.A.L.; Design – O.O., A.A.R., S.Y., T.S., C.Y., A.A.L.; Supervision – O.O., T.S., A.A.L.; Materials – O.O., A.A.R., S.Y., C.Y.; Data collection &/or processing – O.O., S.Y., C.Y.; Analysis and/or interpretation – O.O., T.S., A.A.L.; Literature search – O.O., A.A.L.; Writing – O.O., A.A.L.; Critical review – O.O., A.A.R., S.Y., C.Y., A.A.L.

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