

The Status of CTLA-4 in Colorectal Carcinomas; Relationship with Prognostic Parameters, Her-2 and MMR Proteins

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ABSTRACT

Objective: Colorectal cancer (CRC) is the third most common type of cancer worldwide and the fourth most common cause of cancer-related deaths. Innovative approaches like immunotherapy are necessary for treatment-resistant patients with a poor prognosis. This study aimed to investigate the status of CTLA-4, one of the immune checkpoint molecules, in CRCs.

Material and Methods: A total of 183 resected CRCs were analyzed retrospectively. Immunohistochemical staining was conducted using an anti-CTLA-4 antibody. The relationship between CTLA-4 and prognostic parameters, such as HER2 and microsatellite instability (MSI), was investigated.

Results: One hundred and four of the cases were male, and 79 were female. One hundred fifty-nine of the cases were conventional adenocarcinomas, while 24 were mucinous adenocarcinomas. In 53 of the cases (29%), CTLA-4 showed less than 1% staining in the immune cells, classifying it as negative. In the remaining cases (71%), varying degrees of immune cell staining with CTLA-4 were observed. Conventional adenocarcinomas exhibited a higher expression of CTLA-4 compared to mucinous carcinomas. No statistically significant correlation was found between MSI status and CTLA-4 immune cell staining ($p = 0.572$). CTLA-4 positivity decreased significantly as tumor diameter decreased ($p = 0.029$). There was no correlation between the CTLA-4 immunostaining and median survival time.

Conclusion: CTLA-4 expression was detected in 71% of CRCs. CTLA-4 positivity significantly decreased as tumor diameter decreased. There was no significant association between CTLA-4 expression and MSI or other prognostic parameters. Additionally, no significant correlation was found between CTLA-4 expression and median survival time. Nevertheless, the presence of CTLA-4 expression in the majority of CRCs is promising for anti-CTLA-4 therapy.

Keywords: Colorectal carcinoma, Immunotherapy, Immune checkpoints, CTLA-4

INTRODUCTION

Colorectal cancer (CRC) is one of the leading causes of mortality and morbidity worldwide. It is the third most common cancer globally and the fourth leading cause of cancer-related deaths (1). Approximately 20-25% of CRC cases are diagnosed at the metastatic stage, and an additional 25% of patients develop metastases during the course of their disease (2). Patients with CRC can benefit from early-stage surgical resection and adjuvant chemotherapy. However, effective treatment options are lacking for those with advanced CRC, particularly in cases with metastasis (3). The prognosis for patients with advanced CRC is poor, with a 5-year survival rate of only 12% (4). Novel biologic agents targeting the epidermal growth factor receptor

(EGFR) and vascular endothelial growth factor (VEGF) are the cornerstones of advanced or metastatic CRC treatment. Primary or acquired resistance to these therapies causes CRC patients to seek alternative treatments. Thus, investigating CRC and the status of immunotherapy and immunotherapeutic agents is crucial (5).

The development of immune checkpoint inhibitors has revolutionized antitumor therapies for many cancers, yielding positive prognostic results linked to longer survival for some patients with advanced or metastatic cancer (6). However, immunotherapy is less effective in CRC. Nevertheless, immunotherapy is now considered the new standard treatment for CRC with high microsatellite instability (MSI-H). CRCs with MSI-H have a better response to

immunotherapy. Therefore, it is essential to determine the MSI status of the tumor and the mutational burden of the tumor before starting treatment for CRC (7).

CTLA-4 is a molecular ligand and is part of the inhibitory molecules within the immunoglobulin superfamily. It is transiently expressed on the surface of some activated T cells (8). CTLA-4 is found on the surface of both CD4 and CD8 T cells, binding to B7 ligands on antigen presenting cells (APCs). This binding prevents B7 from attaching to CD28 receptors on T cells, thereby inhibiting T cell activation (5). The expression of CTLA-4 expression is associated with poor prognosis in various tumor types and is believed to play a crucial role in anti-tumor immune responses. In this context, blocking CTLA-4 may help reactivate T cells and restore their capacity to target cancer cells (9).

Ninety-five percent of patients with metastatic CRC who are not MMR deficient (dMMR) or who are microsatellite stable (MSS) face a poor prognosis. When standard treatments such as surgery, chemotherapy, and radiotherapy fail to produce the desired outcomes, innovative approaches like immunotherapy are essential. To this end, our study investigated the expression of CTLA-4, an important immune checkpoint molecule, in CRCs.

MATERIAL and METHODS

This study received approval from the Ethics Committee of Karadeniz Technical University, protocol number 2024/60.

Study Population

The study included 183 resected CRCs from our institution. The patients in our study were diagnosed between 2015 and 2019 and were followed for a period of 5 to 9 years post-diagnosis. Consultations and endoscopic biopsies were excluded. Immunohistochemical markers for MMR were previously examined in all cases.

Immunohistochemical Method and Antibodies

Immunohistochemical staining was performed using the Ventana BenchMark Ultra automated system. Cases were analyzed with an Olympus BX51 microscope. Anti-CTLA-4 antibody 100UL (Abcam / ab237712) at a dilution of 1/200 was used for immunohistochemical evaluation. Previously analyzed archival cases for HER2 and MMR proteins were re-evaluated (Ventana anti-her2/neu (4B5) rabbit monoclonal primary antibody, Ventana anti-MSH6 (SP93) rabbit monoclonal primary antibody, Ventana anti-PMS2 (A 16-4) monoclonal rabbit primary antibody, Ventana anti-MLH1 (M1) monoclonal rabbit primary antibody, Ventana anti-MSH2 (G219-1129) monoclonal rabbit primary antibody).

CTLA-4 Evaluation Method

Staining of CTLA-4 in tumor-infiltrating immune cells was performed as suggested by Fehrenbacher et al. (In the percentage of tumor area: IC3 $\geq$ 10%, IC2 $\geq$ 5% and <10%, IC1 $\geq$ 1% and <5%, and IC0 <1%) (10).

Immunohistochemical Evaluation of the MMR Proteins (MLH1, MSH2, MSH6 and PMS2)

Immunohistochemical markers were scored according to the College of American Pathologists (CAP) protocol: If none of the markers is lost, there is no or only a low risk of MSI. If one of the markers is lost, there is a possible or high risk of MSI (11).

Immunohistochemical Evaluation of HER2 Protein

Immunohistochemical HER2 staining was evaluated according to the Colon and Rectum Biomarker Reporting of CAP protocol (11).

Evaluation of Tumor Regression Score

In our study, we used the Modified Ryan Scoring System to assess tumor regression scores: Complete response (Score 0), near-complete response (Score 1), partial response (Score 2), and no response (Score 3) (12,13).

Tissue Microarray Preparation

Formalin-fixed and paraffin-embedded tissue samples were used to prepare tissue microarrays. Using a skin biopsy device, tissue cores with a diameter of approximately 3 mm were removed from the tumor samples. Subsequently, a new paraffin block containing nine tissue cores was prepared.

Statistical Analysis

The SPSS 26.0 statistical package program was used for data analysis. Descriptive statistics were presented as frequencies and percentages for categorical variables, and as median (minimum–maximum) for continuous and non-normally distributed data.

The Kolmogorov-Smirnov test or the Shapiro-Wilk test was used to assess the normality of the measurement data. The Mann-Whitney U test was used to compare mean ranks for two independent groups and non-normally distributed data. The Chi-square test was used for the analysis of categorical data. Survival analyses were performed using the Kaplan-Meier method, and differences between groups were evaluated with the log-rank test. Median survival times with standard errors and 95% confidence intervals are reported for each group. The significance level was accepted as $p < 0.05$ for all statistical analyses.

RESULTS

Our study includes a total of 183 cases, consisting of 104 males and 79 females. Of these, 159 were conventional adenocarcinomas, while 24 were mucinous adenocarcinomas. In terms of tumor location, 39 cases were located in the rectum, 55 in the right colon, 84 in the left colon, and 5 in the transverse colon. The average age of the patients was 63.3 years, ranging from 23 to 91 years. The prognostic parameters of the cases are presented in Table I. Twenty-four patients included in our study received neoadjuvant chemoradiotherapy that combines chemotherapy and ra-

diotherapy. The treatment response status of the patients and their tumor regression are presented in Table II. Ninety-six cases showed no lymph node metastases, while eighty-seven cases had between one and thirty-nine lymph node metastases. The classification of lymph node metastases is detailed in Table I.

In 53 cases (29%), CTLA-4 showed less than 1% staining in immune cells and was categorized as negative. In the remaining cases (71%), varying degrees of immune cell staining with CTLA-4 were noted (Figure 1). The status of CTLA-4 staining in immune cells for these cases is shown

Table I: Prognostic parameters of the cases and staining status of immune cells with CTLA-4

Prognostic parameters	n	%	Prognostic parameters	n	%
Tumor grade			Tumor budding		
Grade 1	104	56.8	No	45	24.6
Grade 2	54	29.5	Yes	138	75.4
Grade 3	25	13.7	Infiltrative border		
Tumor extent (pT Category)			No	10	5.5
Submucosa (pT1)	3	1.6	Yes	173	94.5
Muscularis propria (pT2)	21	11.5	Mesenteric tumor nodule		
Pericorectal tissues (pT3)	139	76.0	No	147	80.3
Visceral peritoneum (pT4)	20	10.9	Yes	36	19.7
Lymph node metastasis (pN Category)			Perineural invasion		
No regional lymph node metastasis (pN0)	96	52.4	No	165	90.2
One to three regional lymph nodes are positive (pN1)	53	29.0	Yes	18	9.8
Four to six regional lymph nodes are positive (pN2a)	19	10.4	Venous invasion		
Seven or more regional lymph nodes are positive (pN2b)	15	8.2	No	167	91.3
Distant organ metastasis (pM Category)			Yes	16	8.7
No (pM0)	151	82.5	Lymphovascular invasion		
Yes (pM1)	32	17.5	No	111	60.7
Peritoneal carcinomatosis			Yes	72	39.3
No	175	95.6	Tumor infiltrating lymphocytes (TILs)		
Yes	8	4.4	Sparse	77	42.1
MSI High			Mild	92	50.3
No	145	79.2	Moderate	7	3.8
Yes	38	20.8	Severe	7	3.8
HER2 status			Staining rates of CTLA-4 positive immune cell		
Score 0	143	78.1	< %1	53	29.0
Score 1	25	13.7	≥ %1 - < %5	63	34.4
Score 2	11	6.0	≥ %5 - < %10	41	22.4
Score 3	4	2.2	≥ %10	26	14.2
			Total	183	100.0

in Table I. The correlation between CTLA-4 immune cell positivity and clinical and pathologic parameters is shown in Table III. No significant correlation was found except in patients who received neoadjuvant treatment. In patients receiving neoadjuvant treatment, the expression of CTLA-4 on immune cells increased significantly ($p = 0.023$).

All cases exhibited tumor-infiltrating lymphocytes (TILs), although they occurred sparsely in some cases. All cases with TILs demonstrated varying degrees of CTLA-4 staining. Therefore, when assessing the presence of TILs, they were categorized as sparse, mild, moderate, and severe. The presence of TILs was compared to CTLA-4 staining, but no significant association was found.

Table II: Status of response to treatment in patients treated preoperatively

		Treatment response	Tumor regression score	n	%
Preoperative treatment status	Treatment positive	Complete response	0	0	0
		Near complete response	1	2	1.1
		Partial response	2	11	6.0
		Absent	3	11	6.0
	Treatment negative			159	86.9
	Total			183	100.0

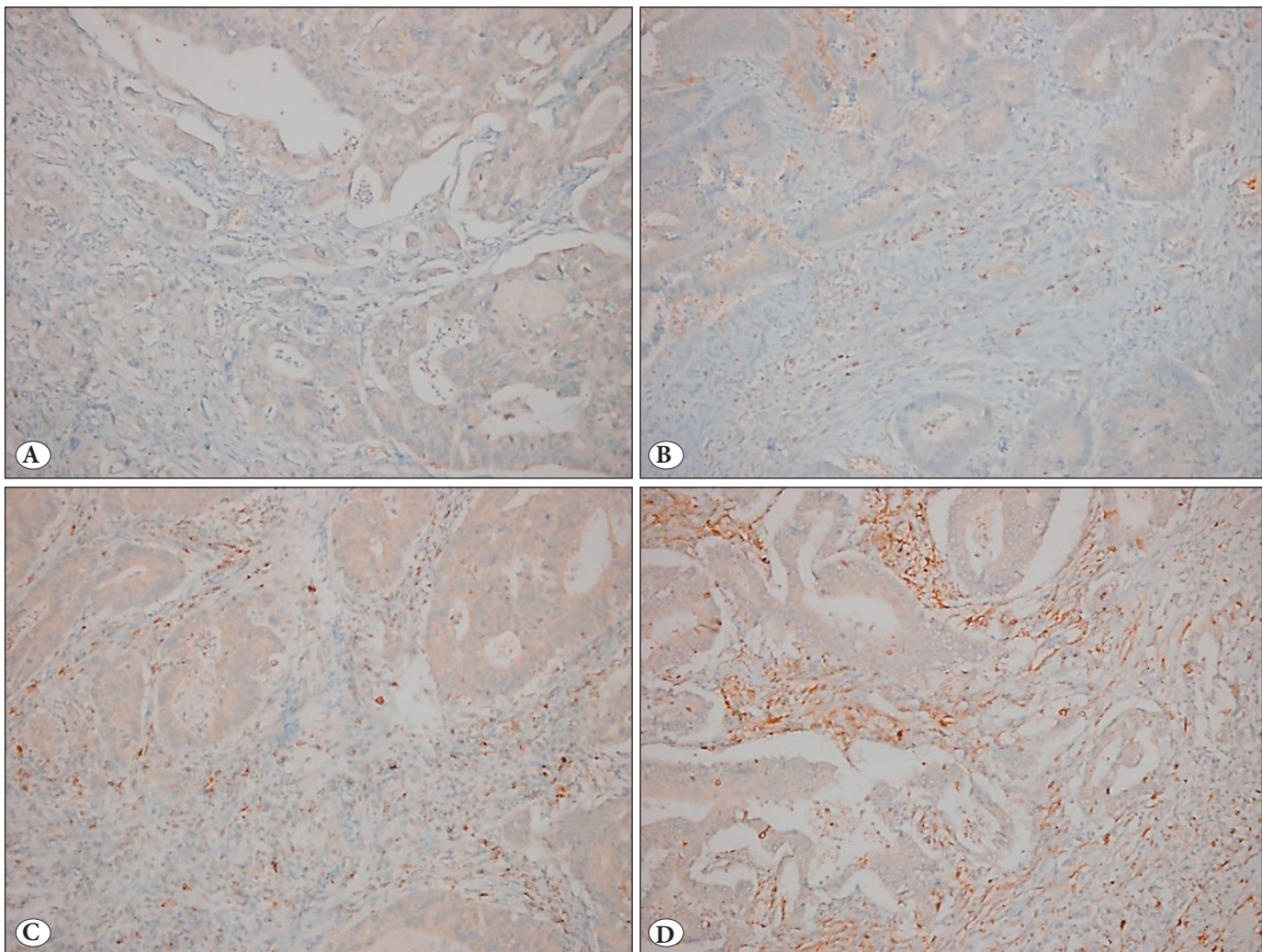


Figure 1: CTLA-4 staining rate. CTLA-4 x 200. **A)** < 1%, **B)** ≥ 1% - < 5%, **C)** ≥ 5% - < 10%, **D)** ≥ 10%

Table III: Relationship between CTLA-4 immune cell positivity and clinical and pathologic parameters.

		CTLA-4 positive immune cell				Total n	P [#]
		<1% n (%) [*]	≥ 1% - < 5% n (%) [*]	≥ 5% - < 10% n (%) [*]	≥ 10% n (%) [*]		
Gender	Female	23 (29.1)	28 (35.4)	18 (22.8)	10 (12.7)	79	0.963
	Male	30 (28.8)	35 (33.7)	23 (22.1)	16 (15.4)	104	
Preoperative treatment	No	50 (31.4)	53 (33.3)	31 (19.5)	25 (15.7)	159	0.023
	Yes	3 (12.5)	10 (41.7)	10 (41.7)	1 (4.2)	24	
Tumor type	AC	43 (25.4)	62 (36.7)	40 (23.7)	24 (14.2)	169	-
	MC	10 (71.4)	1 (7.1)	1 (7.1)	2 (14.3)	14	
	Total	53 (29.0)	63 (34.4)	41 (22.4)	26 (14.2)	183	
Tumor grade	Grade 1	26 (25.0)	40 (38.5)	26 (25.0)	12 (11.5)	104	0.563
	Grade 2	18 (33.3)	17 (31.5)	10 (18.5)	9 (16.7)	54	
	Grade 3	9 (36.0)	6 (24.0)	5 (20.0)	5 (20.0)	25	
Tumor Extent	pT1	1 (33.3)	1 (33.3)	1 (33.3)	0 (0.0)	3	-
	pT2	5 (23.8)	8 (38.1)	5 (23.8)	3 (14.3)	21	
	pT3	38 (27.3)	48 (34.5)	33 (23.7)	20 (14.4)	139	
	pT4	9 (45.0)	6 (30.0)	2 (10.0)	3 (15.0)	20	
Distant organ metastasis	No	44 (29.1)	49 (32.5)	34 (22.5)	24 (15.9)	151	0.436
	Yes	9 (28.1)	14 (43.8)	7 (21.9)	2 (6.3)	32	
Peritoneal carcinomatosis	No	50 (28.6)	62 (35.4)	38 (21.7)	25 (14.3)	175	-
	Yes	3 (37.5)	1 (12.5)	3 (37.5)	1 (12.5)	8	
MSI High	No	39 (26.9)	50 (34.5)	35 (24.1)	21 (14.5)	145	0.572
	Yes	14 (36.8)	13 (34.2)	6 (15.8)	5 (13.2)	38	
HER2 status	0	45 (31.5)	46 (32.2)	32 (22.4)	20 (14.0)	143	-
	1	6 (24.0)	10 (40.0)	4 (16.0)	5 (20.0)	25	
	2	1 (9.1)	5 (45.5)	4 (36.4)	1 (9.1)	11	
	3	1 (25.0)	2 (50.0)	1 (25.0)	0 (0.0)	4	
Tumor budding	No	13 (28.9)	16 (35.6)	11 (24.4)	5 (11.1)	45	0.911
	Yes	40 (29.0)	47 (34.1)	30 (21.7)	21 (15.2)	138	
Infiltrative border	No	2 (20.0)	5 (50.0)	3 (30.0)	0 (0.0)	10	-
	Yes	51 (29.5)	58 (33.5)	38 (22.0)	26 (15.0)	173	
Mesenteric tumor nodule	No	43 (29.3)	49 (33.3)	35 (23.8)	20 (13.6)	147	0.770
	Yes	10 (27.8)	14 (38.9)	6 (16.7)	6 (16.7)	36	
Perineural invasion	No	48 (29.1)	57 (34.5)	37 (22.4)	23 (13.9)	165	0.992
	Yes	5 (27.8)	6 (33.3)	4 (22.2)	3 (16.7)	18	
Venous invasion	No	51 (30.5)	54 (32.3)	37 (22.2)	25 (15.0)	167	0.179
	Yes	2 (12.5)	9 (56.3)	4 (25.0)	1 (6.3)	16	
Lymphovascular invasion	No	31 (27.9)	34 (30.6)	31 (27.9)	15 (13.5)	111	0.156
	Yes	22 (30.6)	29 (40.3)	10 (13.9)	11 (15.3)	72	
Tumor infiltrating lymphocytes (TILs)	Sparse	23 (29.9)	29 (37.7)	20 (26.0)	5 (6.5)	77	-
	Mild	28 (30.4)	26 (28.3)	19 (20.7)	19 (20.7)	92	
	Moderate	1 (14.3)	4 (57.1)	1 (14.3)	1 (14.3)	7	
	Severe	1 (14.3)	4 (57.1)	1 (14.3)	1 (14.3)	7	
	Total	53 (29.0)	63 (34.4)	41 (22.4)	26 (14.2)	183	

#: Chi-Square Test, * % within the line. **AC: Adenocarcinoma.*** MC: mucinous carcinoma

When evaluating the correlation between tumor type, depth of invasion, peritoneal carcinomatosis, and infiltrative border with CTLA-4 staining status, as well as comparing conventional adenocarcinomas and mucinous carcinomas regarding CTLA-4 expression, no p-value could be reported for either. However, conventional adenocarcinomas exhibited a higher expression of CTLA-4 compared to mucinous carcinomas. Statistical analysis indicated that there was no significant correlation between the number of metastatic lymph nodes and MSI status with the expression of CTLA-4 in immune cells ($p = 0.3970.572$, respectively). Due to the very small number of HER2-positive cases, the relationship between HER2 status and CTLA-4 staining status could not be established.

Additionally, a statistical analysis of the CTLA-4 staining status of immune cells was performed by categorizing them into two groups: positive (staining of immune cells $\geq 1\%$) and negative (staining of immune cells $< 1\%$). The statistical analyses performed for CTLA-4 positivity and negativity yielded no significant results, consistent with the findings presented in Table III. When assessed by tumor type, CTLA-4 positivity was observed in 74.6% of the cases (126/169) in conventional adenocarcinomas and in 28.6% of the cases (4/14) in mucinous adenocarcinomas, with a statistically significant higher rate in conventional adenocarcinomas ($p < 0.001$).

The relationship between tumor diameter and CTLA-4 staining status of immune cells is illustrated in Table IV. Interestingly, CTLA-4 positivity significantly increases as tumor diameter decreases ($p = 0.005$). However, this statis-

tical result may not be reliable in patients undergoing treatment, as such treatment can lead to a reduction in tumor diameter. Consequently, the statistics were recalculated by excluding treated patients. Similarly, CTLA-4 positivity showed a significant increase with a decrease in tumor diameter ($p = 0.029$). In addition, the TIL ratio increased with decreasing mean tumor diameter (TIL ratio/mean tumor diameter (cm): sparse/5.14, mild/4.49, moderate/4.21, severe/3.91).

At the time of the study, 59 patients had succumbed to the disease, with deaths occurring within 1 to 8 years following diagnosis, while the remaining patients were alive. Recurrence was observed in 7 patients within 1 to 4 years of diagnosis, and distant metastasis was detected in 32 patients. When examining the correlation between the CTLA-4 staining status of immune cells and the mean survival time (Table V), no statistically significant relationship was found ($p = 0.374$).

DISCUSSION

Tumor cells produce tumor-associated antigens or novel mutant antigens that stimulate an anti-tumor immune response in both innate and adaptive immune cells (14). Alongside other cell types, these cells that trigger the anti-tumor immune response enhance the inflammatory or immune status of the tumor tissue. Tumor-infiltrating lymphocytes (TILs) consist of a mixture of T lymphocytes, B lymphocytes, NK cells, macrophages, and other innate cells in varying proportions, with T lymphocytes being the most abundant within TILs (9).

Table IV: Comparison of Tumor Diameter According to CTLA-4 Immune Cell Positivity

CTLA-4		Tumor diameter (cm)			p value
		Median	Minimum	Maximum	
Treatment positive	Negative (CTLA-4 $< 1\%$) (n=53)	5.000	1.0	15.0	0.005*
	Positive (CTLA-4 $\geq 1\%$) (n=130)	4.000	0.5	13.0	
Treatment negative	Negative (CTLA-4 $< 1\%$) (n=50)	5.250	1.0	15.0	0.029*
	Positive (CTLA-4 $\geq 1\%$) (n=109)	4.500	1.0	13.0	

*: Mann Whitney U test

Table V: Survival Analysis of CTLA-4 Immune Cell Positivity

CTLA-4	Survival time (Year)					p value
	Median	Std. Error	95% Confidence Interval Lower Bound	95% Confidence Interval Lower Bound		
Negative (CTLA-4 $< 1\%$)	6.457	1.250	4.006	8.907		0.374*
Positive (CTLA-4 $\geq 1\%$)	3.803	0.223	3.365	4.240		

*: Kaplan-Meier with log-rank test.

Numerous studies have shown that the presence of TILs is associated with the survival rates of cancer patients, including those with CRCs. However, the immunosuppressive microenvironment arises from the activation of immunosuppressive cells such as regulatory T cells, myeloid-derived suppressor cells, and the PD-1/PD-L1 and CTLA4 signaling pathways. Finally, the balance between the anti-tumor immune response and immunosuppression enables tumor cells to evade immune surveillance. The objective of immunotherapy is to reactivate immune cells and eliminate tumor cells by preventing their escape from immune surveillance. CTLA-4 expression has been identified as a contributor to poor prognosis in the evaluation of various tumor types. This further establishes CTLA-4 as a crucial factor in assessing treatment response within tumors (15). Therefore, inhibiting CTLA-4 enables access to reactive T cells and restores their capacity to attack cancer cells.

Some studies using immunohistochemistry and Western blot have shown that CTLA-4 is expressed in TILs and tumor cells and protects them from immune responses (16,17). In the immunohistochemical study conducted by Narayanan et al. using the monoclonal mouse antibody CTLA4 (IgG1), CTLA-4 overexpression was observed in 43.3% of the tumors. The majority of poorly differentiated tumors in the study exhibited a strong positive (3+) staining intensity, while the majority of well-differentiated tumors displayed a weak positive (1+) staining intensity (18). In our study, we observed cytoplasmic staining in tumor cells. Weak cytoplasmic staining was noted in 109 cases, moderate staining in 8 cases, and strong staining in 1 case, while 65 cases showed no staining. This staining is non-specific and artifactual since it is specific to lymphoid tissue and localizes to the cell membrane.

Derakhshani et al. found in their study using real-time PCR analysis that the expression of CTLA-4 was increased in CRC tissue compared to adjacent non-tumor tissue, although no statistical significance was observed. In our study, there is a limited number of cases representing normal colon mucosa due to the microarray preparation method. In these cases, no staining was seen in the normal colonic mucosa. Derakhshani et al. also determined in their research that CTLA-4 expression has no significant prognostic value in predicting overall survival for CRC patients (19). Our results likewise showed no correlation between overall survival and CTLA-4 expression.

The expression of CTLA-4 in many tumors has been linked to poor prognosis. Liu et al. further demonstrated that CTLA-4 overexpression, as assessed by immunohistochemistry and Western blot, was associated with decreased

overall survival in glioma patients. They also correlated CTLA-4 overexpression with a higher tumor grade (16). In contrast, in the study conducted by Montoyo-Pujol et al. with the Real-Time PCR System, CTLA-4 expression levels were reported as an important independent factor for a favorable prognosis in breast cancer patients (20). However, our study found no significant association between tumor grade and CTLA-4 expression CRCs.

In the current study, we compared CTLA-4 expression with pathological parameters such as lymphovascular invasion, perineural invasion, and venous invasion, as shown in Table III. However, no statistically significant correlation was found between CTLA-4 and these parameters. While the literature is abundant with studies exploring the therapeutic use and prognostic significance of CTLA-4, detailed comparisons between CTLA-4 and specific pathological parameters are lacking.

In their study using Next Generation Sequencing data from the Cancer Genome Atlas, Kitsou M et al. found that CTLA-4 expression is increased in CRCs and stated that high CTLA-4 expression is associated with better survival linked to an effective immune response (21). Similarly, several studies indicate a benefit in terms of progression-free survival, overall survival, and overall response rate for patients who received anti-CTLA-4 treatment compared to those who did not receive treatment (22). In our study, the majority of CRCs (71%) had CTLA-4 expression ($\geq 1\%$). This also suggests that most patients could benefit from anti-CTLA-4 therapy.

Microsatellite instable CRCs in particular respond poorly to chemotherapy and are thus excellent candidates for immunotherapy with CTLA-4 inhibitors (22). Most patients with metastatic CRC of the MSI-H phenotype can benefit from treatment with nivolumab and ipilimumab, and this combination is well tolerated. However, it remains unclear whether combination therapy is superior to single-agent therapy (9). Furthermore, anti-CTLA-4 drugs are only approved as combination therapy for microsatellite instable CRC and are still awaiting approval for standalone use (22).

In recent years, TILs have proven to be a significant prognostic factor (23). Increased tumor budding (TB) and decreased TILs are associated with a worse prognosis in classical adenocarcinoma. Cabuk et al. have reported that there was no significant association between TILs and overall survival (OS) (24). In their Next Generation Sequencing study, Kitsou et al. reported that high expression of immune checkpoint molecules was associated with TIL load (21). The simultaneous increase in CTLA-4 expression and

TIL ratio with decreasing tumor size is an important finding in our study. A few studies comparing CTLA-4 expression and tumor size are found in the literature. In a study by Fu *et al.* using the Real-Time PCR System, the expression levels of PD-1 and CTLA-4 showed statistically significant differences across various immune tissues in relation to different tumor sizes. In particular, it was reported that CTLA-4 expression increases as tumor size decreases in the Colon26 animal model (25). In the study of Zhang *et al.*, the high TIL level was significantly correlated with small tumor size (26). In conclusion, our study supports the positive prognostic significance of smaller tumor size, as indicated by the higher TILs ratio and CTLA-4 expression.

Recent studies have indicated that conventional cancer treatments such as chemotherapy, radiotherapy, or chemoradiotherapy alter the “immune compartment” of the tumor microenvironment. The changes include density, immunological type of TILs and expression patterns of different immune checkpoints (27). In our study, CTLA-4 expression was found to increase significantly in the group receiving neoadjuvant treatment. In the study conducted by Zhang *et al.*, immunohistochemistry was used to assess the effects of two different neoadjuvant therapy methods: chemotherapy and chemoradiotherapy. Similarly, the expression level of CTLA-4 in both groups was significantly higher. However, there was no significant relationship between CTLA-4 levels and the quality of response to the therapies (28).

In our study, conventional adenocarcinomas exhibited higher expression of CTLA-4 compared to mucinous carcinomas, although no statistically significant association was found. A comparison of CTLA-4 expression in immune cells has not been reported in the literature.

The limitations of our study include the small tumor area analyzed due to the microarray method, the lack of representation of normal colonic mucosa, the expression of MMR proteins not confirmed by molecular methods, and potentially a small sample size. A significant limitation of our study is also the disproportionate distribution of tumor stages within the cohort, which is heavily skewed toward advanced stages. Of the 183 cases, only 1.6% (n=3) consisted of early-stage (pT1) tumors, while the vast majority (98.4%) comprised advanced-stage (pT2, pT3, and pT4) cases. Although the literature suggests that expression levels of immune checkpoint molecules may be associated with tumor progression, the concentration of nearly all cases in the advanced stages in our study may have limited the discriminatory power and variance of the statistical analyses. This distribution is considered the primary explanation

for the lack of a statistically significant correlation between CTLA-4 expression and most prognostic parameters or survival time. The numerical imbalance between early and advanced-stage cases makes it challenging to fully capture the potential dynamic changes of CTLA-4 across different tumor stages. Future comparative studies involving larger and more balanced cohorts, specifically with greater representation of early-stage cases, are essential to clarify the prognostic value of CTLA-4.

CONCLUSION

CTLA-4 expression was observed in over 71% of CRCs, exceeding 1%. It was not significantly associated with MSI or most prognostic parameters. Due to the limited number of cases, it was not possible to assess the relationship between HER2 status, other prognostic parameters, and CTLA-4 expression. Furthermore, no significant correlation was found between CTLA-4 expression and median survival time. Nevertheless, the high expression of CTLA-4 in the majority of CRCs indicates that anti-CTLA-4 therapy may hold promise as a treatment option.

Conflict of Interest

We declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authorship Contributions

Concept: **IS**, Design: **IS**, **SAM**, Data collection and/or processing: **AA**, Analysis and/or interpretation: **ZSY**, Literature search: **ZTU**, **GT**, Writing: **IS**, **ZGY**, Approval: **GT**, **ZTU**.

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