Immunohistochemical expression of CD8+ tumor-infiltrating lymphocytes and Foxp3 expression in colorectal carcinoma

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Background/aim
Colorectal cancer (CRC) is third among diagnosed tumors (6.1%) and second according to mortality (9.2%). Disease prognosis is determined not only by the histologic and molecular features of the tumor but also by the host response. Histologic distributions of tumor-infiltrating lymphocytes (TIL) in the microenvironment can be correlated with staging and prognosis of CRC patients. Abundance of CD8+ T lymphocytes has been associated with good prognosis in different types of solid tumors. The association between tumor cell expression of FoxP3 and tumor infiltration by FoxP3-expressing T lymphocytes with prognosis is still controversial. The aim of this study is to evaluate CD8+ TILs and expression of FoxP3 in CRC and to correlate their expression with patients' clinicopathological parameters.

Materials and methods
Tumor paraffin blocks and clinicopathological data of 60 patients with CRC were obtained from the Pathology Department at Cairo University. The density of CD8 TILs and FoxP3 expression was assessed immunohistochemically and evaluated by image analysis in CRC specimens using area percentage parameter. The CD8+ cell tumor infiltrate and FoxP3 expression were classified into scanty, moderate, and abundant.

Results
CD8+ TIL in the present study was insignificantly correlated with the clinicopathological parameters, and no correlation was detected between FoxP3 and CD8 expression ($P>0.05$). However, FOXP3 expression was significantly correlated with tumor grade, nodal status, distant metastasis, tumor stage, and Dukes' classification ($P<0.01$).

Conclusion
The presence of FoxP3 expression in CRC correlates with favorable pathological prognostic parameters. Cancer colon progression is influenced by host immune response. More studies are needed to assess the role of tumor microenvironment in CRC prognosis.

Keywords:
CD8+ tumor-infiltrating lymphocyte, colorectal cancer, FoxP3 expression, immunohistochemistry

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Introduction

Lung cancer is the most common cancer in both sexes (11.6% of the total cases), followed by breast cancer in females (11.6%) and prostate cancer in males (7.1%). Colorectal cancer (CRC) is third among diagnosed tumors (6.1%) and second according to mortality (9.2%). By the year 2035, the total number of deaths from rectal and colon cancer will increase by 60 and 71.5%, respectively [1]. These values may differ from country to country depending on the degree of economic development. Therefore, the disease is widely recognized as a marker of the country's socioeconomic development [2]. In Egypt, CRC represented the seventh most common cancer and the third most common male neoplasm and fifth most common female neoplasm [3].

During the last decade, there has been a progressive increase in our understanding of the tumor microenvironment, which enhances the recognition of main factors of immune response to tumors. The most important and prognostic factor in CRC are tumor-infiltrating lymphocytes (TIL), which are heterogeneous populations of T lymphocytes present in the tumor microenvironment [4]. Disease prognosis is determined not only by the histologic and molecular features of the tumor but also by the host response, particularly the immune response. Inflammatory cell

density is higher in tumor tissue than in adjacent normal tissue in breast cancer patients [5]. Histologic evaluation of TILs in the tumor microenvironment can be correlated with the clinical stage and prognosis in CRC patients [6].

In this regard, the presence of CD8+ T lymphocytes has been associated with favorable prognosis in different types of solid tumors [7,8]. This T lymphocyte population mediates antitumor activity through antigen-specific cytotoxicity and by producing antitumor cytokines, namely IFN-γ and TNF-α [9,10]. On the other hand, increased tumor infiltration by FoxP3-expressing T lymphocytes has been associated with decreased survival of patients with different types of cancer, including the breast [11], lung [12], and cervical cancers [13]. However, this association is not seen in all cancer types, as FoxP3+ T lymphocyte infiltrates have been associated with good prognosis in other cancers, such as head and neck cancers [14]. Other studies have found that Foxp3, besides being a reliable marker of Treg cells, is also expressed by tumor cells and normal tissue cells, where it exerts a tumor-suppressing function [15,16].

As such, much attention has been given to characterizing the immune contexture of tumors and determining the relationship of immune populations within the tumor microenvironment to clinical behavior, prognosis, and therapeutic response. The aim of this study was to evaluate CD8+ TILs and the expression of FoxP3 in CRC and to correlate their expression with patients' clinicopathological parameters.

Materials and methods

Sampling

The present study retrospectively obtained tumor paraffin blocks and reviewed the database of 60 cases that underwent curative surgery for CRC from the Pathology Department, National Research Centre. The personal data of the patients were replaced by numerical codes for privacy and confidentiality.

Ethical consideration

The present study was conducted with the Code of Ethics of the World Medical Association, according to the principles expressed in the Declaration of Helsinki. This study was approved by the Local Ethics Committee of National Research Centre, Cairo, Egypt with approval number 19/027. The used materials were derived from archived tissue samples embedded in paraffin blocks. The blocks and required data were collected from the patients' reports after approval of the head of the lab and were anonymized.

Inclusion criteria

Patients having CRC with no other cancers or diseases, such as acute infection or diabetes, and did not receive previous radiotherapy or chemotherapy at the time of the surgery.

Exclusion criteria

Patients who had other cancers or diseases, such as acute infection or diabetes, and those who had received previous radiotherapy or chemotherapy at the time of the surgery.

Tissues

Three sections (4–5 μm thick) were cut from each block. One section was stained with hematoxylin and eosin for histopathologic evaluation. The other two sections were mounted on a positively charged glass slide for immunohistochemical staining. Histopathological characteristics were reviewed. Histological diagnosis was established and verified by two pathologists. Clinicopathologic data were retrospectively collected from the medical records and are summarized.

Immunohistochemical staining

Immunohistochemical staining was conducted on paraffin-embedded sections by anti-FOXP3 and CD8 antibodies. The slides were incubated at 37°C overnight for accurate adhesion of the section of the slide. For CD8 staining deparaffinization, rehydration, and epitope retrieval were performed in the Pt link retrieval system (Dako, Copenhagen, Denmark). The staining steps and incubation times were preprogrammed into the an AautoStainer Link software (Dakoomnis, Copenhagen, Denmark). Diluted primary antibodies against CD8 (Thermo Fisher Scientific, Runcorn, Cheshire, UK) and horseradish peroxidase labeled secondary antibody (Thermo Fisher Scientific) were used. For negative control, the primary antibody was replaced by normal mouse serum. Diaminobenzidine was used for color development and hematoxylin as a counter stain. For Foxp3 staining tissue sections were dewaxed, and the antigens were exposed by citrate treatment (pH 6.0). Sections were then incubated in 0.3% H_2O_2 at room temperature and washed with distilled water and 0.1 M phosphate-buffered saline before an overnight incubation in 10% goat serum and primary antibody (1 : 200, mouse anti-human FOXP3 monoclonal, produced by Abcam, purchased from Wuhan Amyjet Scientific) at 4°C. Subsequently, sections

were treated with the secondary antibody (1 : 300, goat anti-mouse IgG polyclonal antibody). Staining color was developed with the diaminobenzidine substrate [diaminobenzidine color kit (Beijing Zhongshan Golden Bridge Biotechnology, Beijing. China)]. Tissues were counterstained with hematoxylin. Phosphate-buffered saline was used as negative control for the primary antibody.

Image analysis immunoscoring

Immunostaining was visualized and photographed under a light microscope Olympus CX-41 with DP 12 Olympus Digital Camera (Olympus Optical Co. Ltd, Tokyo, Japan). Quantitative evaluation was performed by screening the sections and selecting at least five different fields with high-density TILs. The density of CD8+ TIL and the positive expression of FoxP3 in tumor cells and TILs were determined using the Leica Q_win 500 image analysis system (LEICA Imaging Systems Ltd, Cambridge, England), which consists of Leica DMLB microscope with JVC color video camera attached to a computer system. We selected five tumor fields with the highest expression and assessed the area percentage of the positive cells at high magnification (×200). We detected the positively stained cells, which were masked automatically by a blue mask called the binary image. We used the measure field software program that automatically measures the area percentage of the detected features in the binary image and then displays the results in a table form. The immune score for FOXP3-positive cells and CD8+Tcells was evaluated according to Cho *et al.* [17] into scanty, moderate, and abundant expression. As for CD8, area percentage from 0 to 1 were scored as scant, 1 to 2 as moderate, and more than 2 as abundant. As for FoxP3, area percentage from 0 to 10 as scant, 10 to 20 as moderate, and more than 20 as abundant.

Statistical analysis

Data were coded using the Statistical Package SPSS version 25 for windows (IBM, New York, USA). Data were recorded using mean, SD, median, minimum, and maximum in quantitative data and using frequency (count) and relative frequency (percentage) for categorical data. Comparisons between quantitative variables were done using the nonparametric Mann–Whitney tests test for independent samples for comparing two groups and Kruskal–Wallis for comparing more than two groups. *P* values less than 0.05 were considered as statistically significant. Differences between groups were analyzed using the χ^2 test. *P* values of less than 0.05 were considered to indicate statistical significance.

Results

The study included 60 specimens from CRC patients; 32 (53.33%) of them were males and 28 (46.67%) were females. The age of the study population ranged from 22 to 83 years. Thirty-three (55%) of them were less than 60 years and 27 (45%) more than or equal to 60 years. The tumor size in cases included in the study ranged from 2 to 14 cm, 21(35%) of them were less than 4.5 in the largest dimension, and 39 (65%) were more than or equal to 4.5. Twenty-one (35% of the tumors) tumors were in the right colon, 22 (36.67%) were in the left side of the colon, and 17 (28.33%) were in the rectum. Forty-one (68.33%) cases were of adenocarcinoma type and 19 (31.67%) were mucinous carcinoma (Table 1).

Histopathological parameters of the studied cases revealed that none of the included tumors were of grade I (well differentiated); 29 (46.33%) were of grade II (moderately differentiated); and 31 (51.67%) were of grade III (poorly differentiated). One (1.67%) of the included cases was T_1 (tumor limited to the mucosa and submucosa), six (10%) cases were T_2 (tumor extended to muscular propria), while the majority of the cases (35, 58.33%) were T_3 (tumor invasion of precolonic fat), and 18 (30%) were T4 (tumor invasion of the adjacent structure). No lymph node metastasis (N_0) was detected in 27 (45%) of the studied cases, 16 (26.67%) of the cases showed metastases in three or less regional lymph nodes (N_1), and 17 (28.33%) of the cases showed metastases in four or more regional lymph nodes (N2).. The majority of the included cases in this study (53; 88.83%) had no distance metastases (M0) and only seven (11.67%) of the cases show distant metastases (M1). Six (10%) of the study cases were stage I, 21 (35.00%) of the cases were stage II, 26 (43.33%) of the cases were stage III, and seven (11.67%) were of stage IV. None of the studied cases were Dukes' class A, 28 (46.67%) were classified B, 25 (41.67%) were classified C, and seven (11.6%) cases were of class D with significant difference (Table 1).

Immunoscoring of CD8+ tumor-infiltrating lymphocytes

Twenty-two (41.66%) of the studied tumors showed scanty CD8+ T cell infiltration with area % of positive staining ranging from 0.0392 to 0.949 and mean of 0.495±0.262. Sixteen (26.67%) of the tumors in this study showed moderate CD8+ T cell infiltration with area % of positive staining ranging from 1.084 to 1.978 and mean of 1.514±0.321. Nineteen (31.67%) of the studied tumors showed abundant CD8+ T cell

Table 1 Clinicopathological characteristics of the studied cases

Clinicopathological features	*N*=60 (100%) [*n* (%)]
Sex	
Male	32 (53.33)
Female	28 (46.67)
Age (years)	
<60	33 (55)
≥60	27 (45)
Tumor size	
<4.5 cm	21 (35)
≥4.5 cm	39 (65)
Tumor site	
Right	21 (35)
Left	22 (36.67)
Rectum	17 (28.33)
Tumor type	
Adenocarcinoma	41 (68.33)
Mucinous	19 (31.67)
Tumor grade	
I	0
II	29 (48.33)
III	31 (51.67)
T classification	
T1	1 (1.67)
T2	6 (10)
T3	35 (58.33)
T4	18 (30.00)
N classification	
N0	27 (45.00)
N1	16 (26.67)
N2	17 (28.33)
M classification	
M0	53 (88.33)
M1	7 (11.67)
Stage	
I	6 (10.00)
II	21 (35.00)
III	26 (43.33)
IV	7 (11.67)
Dukes' classification	
A	0
B	28 (46.67)
C	25 (41.67)
D	7 (11.67)

infiltration with area % of positive staining ranging from 2.08 to 5.507 and mean of 3.833±1.710 with highly significant difference (Table 2).

No significant correlation could be detected between CD8+ TIL and sex, age, tumor size, and site ($P>0.05$). Although, 39.02% of adenocarcinomas showed abundant CD8+ T cell infiltration and 15.79% of mucinous carcinomas showed abundant CD8+ T cell infiltration; the difference was insignificant ($P>0.05$). There was no significant correlation that could be detected between CD8+ TIL and all histopathological parameters in the studied cases ($P>0.05$) as shown in Table 3 and Fig. 1.

Immunoscoring of FOXP3-positive expression

Seven (11.67%) of the studied tumors showed scanty FOXP3-positive staining both in tumor cells and T cell infiltration with area % of positive staining ranging from 1.5145 to 7.44 and mean of 0.495±0.262.

Twenty-four (40.00%) of the studied tumors showed moderate FOXP3-positive expression with area % of positive staining ranging from 10.1832 to 17.627 and mean area of 13.135±2.045.

Twenty-nine (48.33%) of the studied tumors showed abundant FOXP3-positive expression with area% of positive staining ranging from 20.1285 to 26.684 and mean area of 22.812±2.070 (Table 2).

No significant correlation could be detected between Foxp3-positive expression and sex, age, tumor size, site, or type ($P>0.05$). All grade II cases showed abundant Foxp3-positive expression and most of grade III cases (77.42%) showed moderate FoxP3-positive expression both in tumor cells and infiltrating T cells with highly significant correlation ($P<0.001$). There was insignificant correlation between T classification and Foxp3-positive expression ($P>0.05$). There was highly significant correlation between lymph node status, distant metastasis, tumor stage, Dukes' classification, and Foxp3-positive expression ($P<0.001$), as shown in Table 4 and Fig. 2. There was insignificant correlation between CD8+T cell infiltration and FoxP3-positive expression.

Discussion

CRC is one of the most prevalent malignant neoplasms. Depending on the location, type of cancer or gender, it is ranked second to fourth in terms of incidence in the world [18]. Tumor progression and the efficacy of immunotherapy are strongly influenced by the composition and abundance of immune cells in the tumor microenvironment [19]. TILs have a major role recruitment, maturation, and activation of immune cells that suppress tumor growth. Tumor infiltration by T lymphocytes is a prognostic factor for CRC outcome, independent of traditional prognostic indicators [20,21]. Previous studies have demonstrated that the type, density, and site of TILs in primary tumors are prognostic for disease-free survival and overall survival from CRC and hints at

Table 2 Immunoscoring of CD8-positive tumor infiltration lymphocyte and FoxP3 in colorectal carcinoma

Expression in CRC cases	Scanty	Moderate	Abundant	*P* value
CD8+ T cells				<0.001**
Number of patients (%)	25 (41.61)	16 (26.67)	19 (31.67)	
Range	0.039–0.949	1.084–1.978	2.08–5.507	
Area (mean±SD)	0.495±0.262	1.514±0.321	3.833±1.710	
FoxP3+expression				<0.001**
Number of patients (%)	7 (11.67)	24 (40.00)	29 (48.33)	
Range	1.5145–7.44	10.1832–17.627	20.1285–26.684	
Area (mean±SD)	4.623±2.267	13.135±2.045	22.812±2.070	

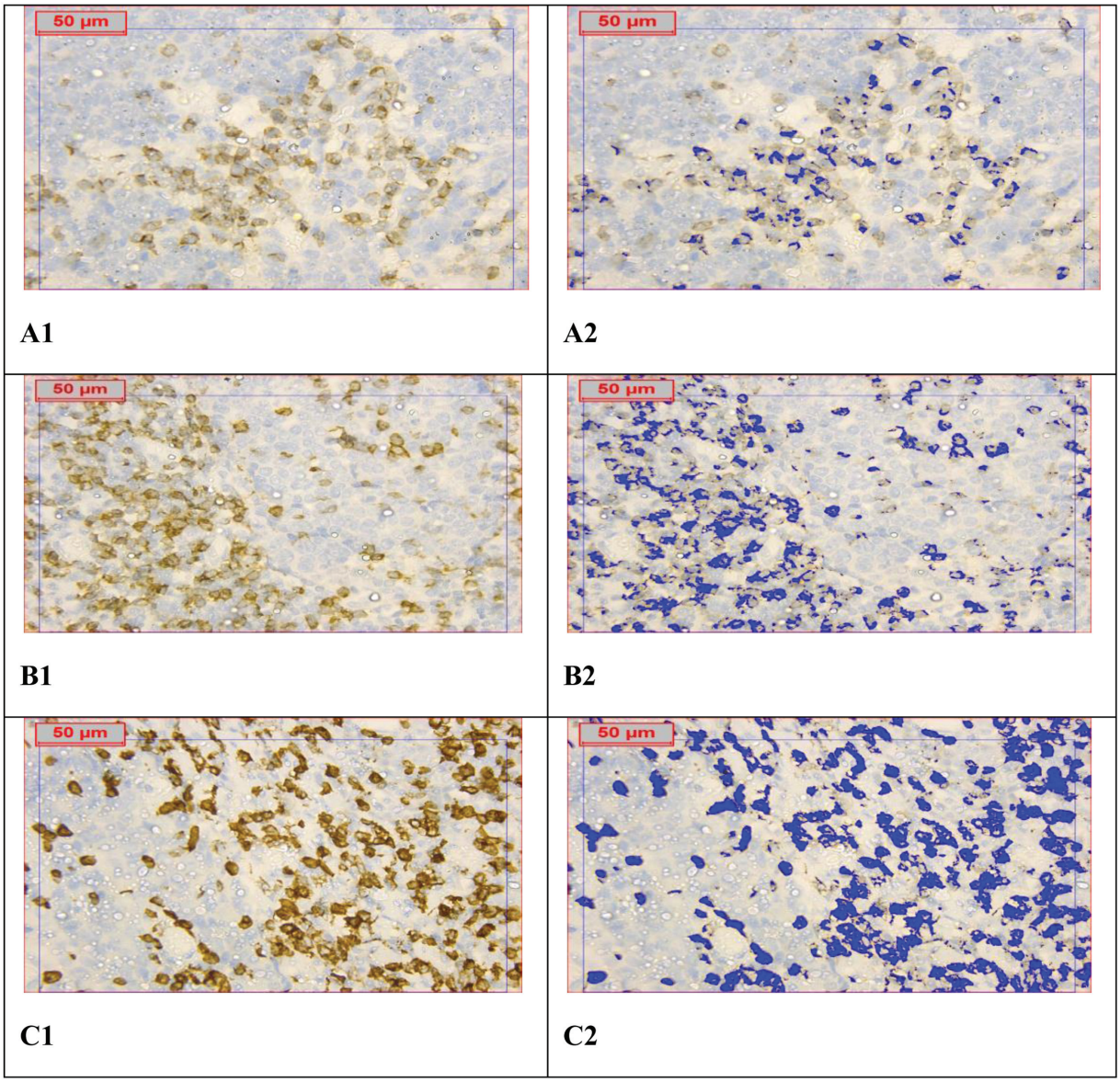
CRC, colorectal cancer. **Highly significant correlation at *P* value less than 0.001, using Kruskal–Wallis test.

Table 3 Correlation between CD8+ T cell infiltration in CRC and clinicopathologic parameters

CD8+cells	Scanty (*N*=25) [*n* (%)]	Moderate (*N*=16) [*n* (%)]	Abundant (*N*=19) [*n* (%)]	Total (*N*=60) [*n* (%)]	*P* value
Sex					>0.05*
Male	12 (42.86)	7 (25.00)	9 (32.14)	28 (100)	
Female	13 (40.63)	9 (28.13)	10 (31.25)	32 (100)	
Age (years)					>0.05*
<60	15 (45.45)	9 (27.27)	9 (27.27)	33 (100)	
≥60	10 (37.04)	7 (25.93)	10 (37.04)	27 (100)	
Tumor size					>0.05*
<4.5 cm	11 (52.38)	4 (19.05)	6 (28.57)	21 (100)	
≥4.5 cm	14 (35.90)	12 (30.77)	13 (33.33)	39 (100)	
Tumor site					>0.05*
Right	8 (38.10)	7 (33.33)	6 (28.57)	21 (100)	
Left	10 (45.45)	7 (31.82)	5 (22.73)	22 (100)	
Rectum	7 (41.18)	2 (11.76)	8 (47.06)	17 (100)	
Tumor type					>0.05*
Adenocarcinoma	16 (39.02)	9 (21.95)	16 (39.02)	41 (100)	
Mucinous carcinoma	9 (47.37)	7 (36.84)	3 (15.79)	19 (100)	
Tumor grade					>0.05*
I	0	0	0	0	
II	14 (48.28)	8 (27.59)	7 (24.14)	29 (100)	
III	11 (35.48)	8 (25.81)	12 (38.71)	31 (100)	
T classification					>0.05*
T1	0	0	1 (100)	1 (100)	
T2	2 (33.33)	2 (33.33)	2 (33.33)	6 (100)	
T3	15 (42.86)	9 (25.71)	11 (31.43)	35 (100)	
T4	8 (44.44)	5 (27.78)	5 (27.78)	18 (100)	
N classification					>0.05*
N0	13 (48.15)	6 (22.22)	8 (29.63)	27 (100)	
N1	5 (31.25)	6 (37.50)	5 (31.25)	16 (100)	
N2	7 (41.18)	4 (23.53)	6 (35.29)	17 (100)	
M classification					>0.05*
M0	23 (43.40)	13 (24.53)	17 (32.08)	53 (100)	
M1	2 (28.57)	3 (42.86)	2 (28.57)	7 (100)	
Stage					>0.05*
I	2 (33.33)	2 (33.33)	2 (33.33)	6 (100)	
II	12 (57.14)	4 (19.05)	5 (23.81)	21 (100)	
III	9 (34.62)	7 (26.92)	10 (38.46)	26 (100)	
IV	2 (28.57)	3 (42.86)	2 (28.57)	7 (100)	
Dukes' classification					>0.05*
A	0	0	0	0	
B	13 (46.43)	7 (25.00)	8 (28.57)	28 (100)	
C	10 (40.00)	6 (24.00)	9 (36.00)	25 (100)	
D	2 (28.57)	3 (42.86)	2 (28.57)	7 (100)	

*Insignificant results at *P* value >0.05 using the Kruskal–Wallis Test.

Figure 1



Immunohistochemical staining of CD8+ TIL in colorectal carcinoma and their binary images by Leica Qwin 500 image analysis system: (A1 and A2) scanty expression, (B1 and B2) moderate expression, and (C1 and C2) abundant expression of CD8 immunohistochemical staining (×400). TIL, tumor-infiltrating lymphocyte.

a fundamental function of the immune system in the tumor microenvironment [22,23].

Several studies have shown that high infiltrations of CD3+ and/or CD8+ T lymphocytes within CRC tumors and their invasive margins were associated with early stages of the disease (TNM stages I–II) and other good prognostic indicators, including absence of lymph node metastasis and distant metastasis [24,25]. Previous studies has shown that while CD8+ T lymphocytes are directly capable of killing tumor cells and positively affect prognosis in a wide range of tumors [26,27], several other studies have shown no such correlation with prognosis [28,29]. As such, it is possible that the tumor microenvironment could modulate the function of CD8+ T lymphocytes, and that this effect may depend on environmental variables such as the microbiome [30,31] or the tumor-inflammatory status [32].

In the present study, we investigated TILs as a potential marker of prognosis in CRC and correlated the findings with clinicopathological variables.CD8+ TIL in the present study were insignificantly correlated with the clinicopathological parameters. Similarly, Barbosa *et al.* [33] were not able to find any correlation between the presence and extent of intratumor T lymphocyte infiltrates with the clinical

Table 4 Correlation between Foxp3-positive expression and clinicopathological parameters of colorectal cancer cases

Foxp3-positive expression	Scanty (*N*=7) [*n* (%)]	Moderate (*N*=24) [*n* (%)]	Abundant (*N*=29) [*n* (%)]	Total (*N*=60) [*n* (%)]	*P* value
Sex					>0.05
Male	4 (14.29)	12 (42.86)	12 (42.86)	28 (100)	
Female	3 (9.38)	12 (37.50)	17 (53.13)	32 (100)	
Age (years)					>0.05
<60	5 (15.15)	13 (39.39)	15 (45.45)	33 (100)	
≥60	2 (7.41)	11 (40.74)	14 (51.85)	27 (100)	
Tumor size					>0.05
<4.5 cm	3 (14.29)	11 (52.38)	7 (33.33)	21 (100)	
≥4.5 cm	4 (10.26)	13 (33.33)	22 (56.41)	39 (100)	
Tumor site					>0.05
Right	3 (14.29)	9 (42.86)	9 (42.86)	21 (100)	
Left	3 (13.64)	5 (22.73)	14 (63.64)	22 (100)	
Rectum	1 (5.88)	10 (58.82)	6 (35.29)	17 (100)	
Tumor type					>0.05
Adenocarcinoma	3 (7.32)	17 (41.46)	21 (51.22)	41 (100)	
Mucinous carcinoma	4 (21.05)	7 (36.84)	8 (42.11)	19 (100)	
Tumor grade					<0.001**
I	0	0	0	0	
II	0	0	29 (100)	29 (100)	
III	7 (22.58)	24 (77.42)	0	31 (100)	
T classification					>0.05
T1	0	1 (100)	0	1 (100)	
T2	0	0	6 (100)	6 (100)	
T3	15 (42.86)	9 (25.71)	4 (31.43)	35 (100)	
T4	3 (16.67)	5 (27.78)	10 (55.56)	18 (100)	
N classification					<0.001**
N0	0	1 (3.70)	26 (96.30)	27 (100)	
N1	4 (25.00)	10 (62.50)	2 (12.50)	16 (100)	
N2	3 (17.65)	13 (76.47)	1 (5.88)	17 (100)	
M classification					<0.001**
M0	0	24 (45.28)	29 (54.72)	53 (100)	
M1	7 (100.00)	0	0	7 (100)	
Stage					<0.001**
I	0	0	6 (100)	6 (100)	
II	0	0	21 (100)	21 (100)	
III	0	24 (92.31)	2 (7.69)	26 (100)	
IV	7 (100.00)	0	0	7 (100)	
Dukes' classification					<0.001**
A	0	0	0	0 (100)	
B	0	1 (3.57)	27 (96.43)	28 (100)	
C	0	23 (92.00)	2 (8.00)	25 (100)	
D	7 (100)	0	0	7 (100)	

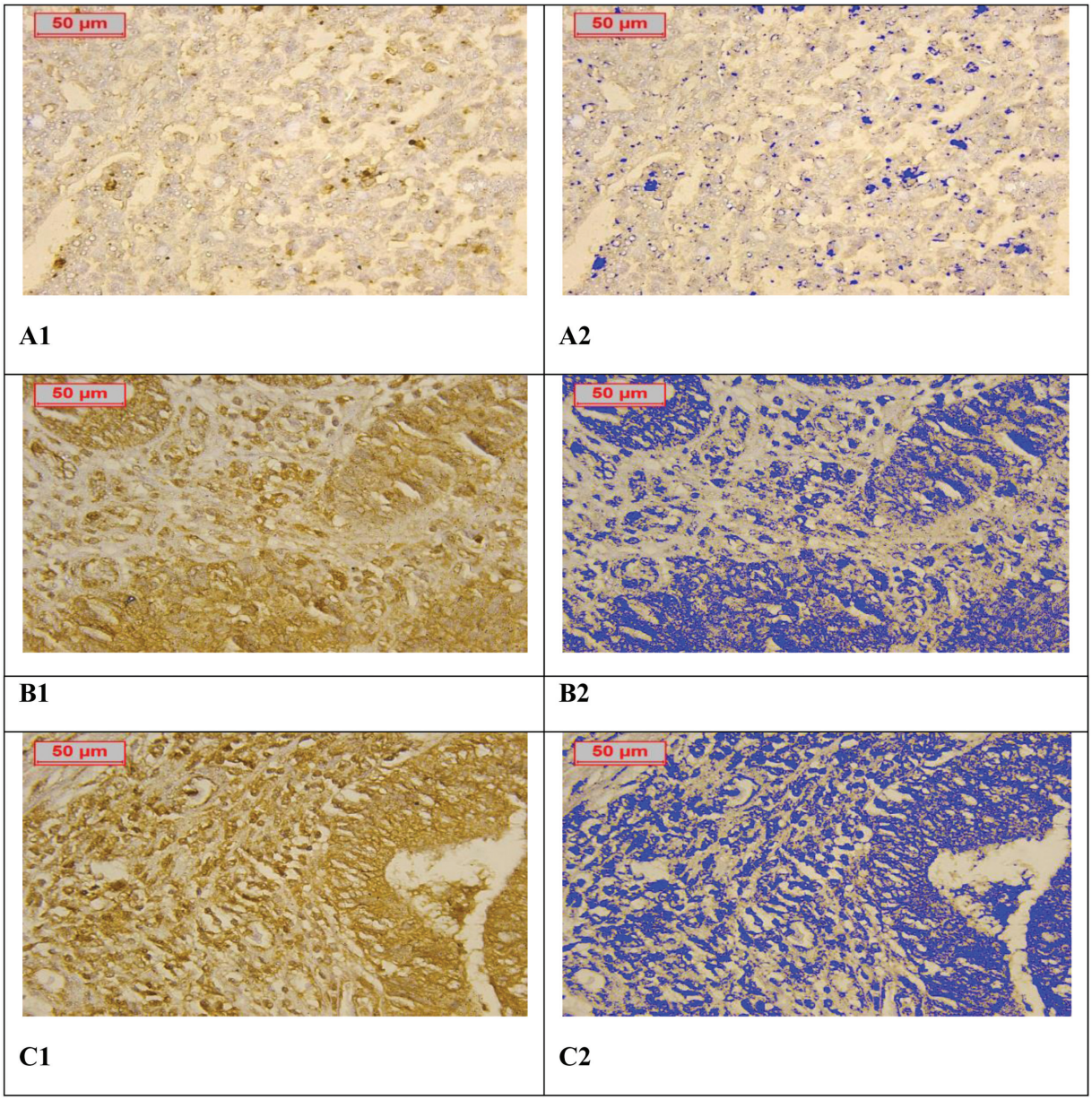
**Highly significant correlation at *P* value less than 0.001 using the Kruskal–Wallis test.

or pathological data of the patients, indicating that intratumor lymphocytes do not influence the pathogenesis of CRC.

Results of the Abdelaal *et al.* [34] study showed that there was no significant correlation between CD4+ and CD8+ cell infiltration of the tumor tissue with any of clinicopathological parameters. Similar results were reported by Matsutani *et al.* [35] who showed no significant association of CD8+ T cells with any of the clinicopathological parameters except significant association of the density of CD8+ T cells and lymph node metastasis. According to Guidoboni *et al.* [36], the presence of CD8+ T cells in the stromal and peritumoral tissue was not correlated with better prognosis, whereas CD8+ T cells infiltrated within cancer cell nests were associated with good survival, independent of the pathologic stage.

Idos *et al.* [37] found that the presence of high CD3+, CD8+, or FoxP3+ T lymphocyte infiltrates were associated with TNM stages I and II, negative lymph nodes, and normal CEA levels. Illustrating that the presence of CD3+, CD8+, or FoxP3+ T

Figure 2



Immunohistochemical staining of FoxP3 in colorectal carcinoma and their binary images by Leica Qwin 500 image analysis system: (A1 and A2) scanty expression, (B1 and B2) moderate expression, and (C1 and C2) abundant expression of (FoxP3) immunohistochemical stain (×400).

lymphocytes in the tumor-invasive margins are associated with good prognostic indicators. TILs have a great role in recruitment, maturation, and activation of immune cells that suppress tumor growth. Tumor infiltration by T lymphocytes is a highly informative prognostic factor for CRC outcome, independent of traditional prognostic indicators [21,38]. Previous studies have demonstrated that the type, density, and site of TILs in primary tumors are prognostic for disease-free survival and overall survival from CRC and hints at a fundamental function of the immune system in the tumor microenvironment [23,39].

Our results showed that high densities of FoxP3 are associated with well-differentiated tumors and absence of lymph node involvement. Most of the included cases without distant metastases showed abundant expression, while all cases with distant metastases showed scanty expression with highly significant difference ($P<0.001$). All stage I and II included cases showed abundant expression of FoxP3. Most of stage III cases showed moderate expression of FoxP3, and all included cases of stage IV showed scanty expression with highly significant difference ($P<0.001$). Most of Dukes' class B cases showed abundant expression of FoxP3; 92.00% of Dukes'

class C cases showed moderate expression; and all cases of Dukes' class D showed scanty expression with highly significant difference ($P<0.001$).Similarly, Sun *et al.* [40] proved that as T stage increased, Foxp3 expression decreased. Also, Foxp3 expression was significantly decreased in the poorly differentiated tumor group. This implies that a high Foxp3 expression is associated with a longer mean survival time. Hu *et al.* [41] found that high density of FoxP3+ Tregs within the tumor especially at the stromal compartment leads to a favorable clinical outcome of CRC, indicating that FoxP3+ Tregs are one of the potential indexes for prognostic prediction and agonists through promoting FoxP3+ Tregs generation, which may be promising in immunotherapy for human CRC. Also, Barbosa *et al.* [33] showed that the high infiltration of FoxP3+ T lymphocytes in tumor margins was associated with TNM stages I–II, normal CEA levels, and, negative lymph nodes. Previous studies have reported that Foxp3 expression in tumor cells have been shown to play an important role in the prognosis of many cancers.

These results are in contrast to that from the study of Ling *et al.* [42], which showed that cell densities in cases with metastases were higher than those without metastasis. Cases with serosal infiltration showed higher FOXP3+ Treg cell densities compared with cases without infiltration and more cell densities in poorly differentiated cases than in well differentiated cases. They also stated that FoxP3+ Tregs infiltrating into different sites (intraepithelium or stroma) seemed to predict differential clinical outcomes, implicating FoxP3+ Tregs may have distinct roles depending on their localization. Hu *et al.* [41] reported that the possible explanation was that FoxP3+ Tregs in stroma mainly inhibited the inflammatory antimicrobial response facilitating tumor progression, whereas FoxP3+ Tregs in the intraepithelium may inhibit antitumor immunity and promote tumor immune evasion maybe through direct contact with tumor cells. The main reason for this controversy in results may be differences in sample size, experimental methods, and statistical analysis.

Conclusion

High expression of FoxP3 is associated with favorable prognostic indicators and can be used as a prognostic biomarker for CRC behavior and outcome. However, its relation to TILs in the CRC tumor microenvironment is still unclarified and requests further and larger scale studies.

Conflicts of interest

No conflict of interest.

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