

## STUDY OF TUMOR MICROENVIRONMENT AND HOST RESPONSE IN COLORECTAL CANCER: IMMUNOHISTOCHEMICAL AND CLINICOPATHOLOGICAL APPROACH

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### ABSTRACT

**Objective:** This study aimed to evaluate the immunohistochemical expression of forkhead boxP3 (Foxp3), CD8, CD68, and CD21 in stroma between tumor cells of colorectal cancer (CRC) patients and examines the relationship between these variables and clinicopathological parameter and patients' prognosis.

**Methods:** In this work, 50 cases of colorectal carcinomas were included and immunohistochemical evaluation of Foxp3, CD8, CD68, and CD21 in tumor tissue samples.

**Results:** Tumor-infiltrating lymphocytes (TILs) including cytotoxic T cells and regulatory T cells as well as tumor-associated macrophages and follicular dendritic cells (FDCs) were studied in the stroma of the tumor using immunostaining technique for CD8, Foxp3, CD68, and CD21, respectively. Cases were followed up. CD8-positive cytotoxic T cells, Foxp3-positive regulatory T cells, and CD21 positive-FDCs were significantly more pronounced in early tumors and those with longer overall survival. On the other hand, CD68 positive macrophages were more encountered in late stage and metastatic tumors as well as tumors with shorter overall survival, but these results not reached the level of significance.

**Conclusion:** We concluded that (TILs) and FDCs are conferring better prognosis in CRCs, they may act synergistically in stimulating a protective immune response in the tumor microenvironment that hinders tumor progression, while the role of tumor-associated macrophages in CRCs is still controversial and needs further studies.

**Keywords:** Colorectal cancer, Lymphocyte subsets, Tumor-associated macrophage, Tertiary lymphoid structure, Survival.

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### INTRODUCTION

Colorectal cancer (CRC) is one of the most common solid cancer affecting both sexes nearly 10% of total cancer cases [1,2]. In Egypt, CRC occupies first rank among digestive system malignancies and fifth rank among total cancers [3]. Mortality related to CRC has descended gradually in many Western countries, which can be imputed to cancer screening programs and early detection. However, rates have continued to increase in our developing countries with poor health-care resources [4,5].

Despite the great amelioration in early diagnosis, the prognosis of CRC patients remains far from satisfaction; this enhances efforts for improvement of new prognostic markers for risk stratification in CRC. The era of tumor microenvironment (TME) is now widely studied for this purpose [6]. Stromal cells can both promote and inhibit tumor cell growth. Now cancer research has recently shifted from studying tumor cells itself to evaluation of cells and mediators encountered in TME [7]. From the wide variety of cells and factors constituting TME, collectively lymphoid neogenesis in the form of lymph node-like structures [8-10] or individually infiltrating immune cells are attracting great attention. These cells are playing a fateful role in tumor progression through a complex interaction with all other components of TME. However, those interactions are still convoluted and argumentative issues were reported on it [11,12]. The association of immune system activity and prognosis in CRC was described for a long time [13]. In this work, we study the tumor-infiltrating lymphocytes (TILs) and its correlation with other clinicopathological parameters in CRC.

### METHODS

The present retrospective study included 50 cases of CRC submitted to pathology then to the Clinical Oncology Department, Faculty of Medicine, University of Alexandria, during the period between January 2013 and December 2016. Follow-up data were obtained by reviewing charts and tumor registry records. Cases with insufficient tumor sample, unavailable clinical information, and pretreated colorectal carcinomas were excluded from the study.

The study was approved from the Ethics Committee before work conduction.

#### Histopathology

Formalin-fixed paraffin-embedded blocks of the included 50 cases of colorectal carcinomas were cut into 5 µm – thick section and stained by H and E for confirmation of the histologic diagnosis, subtyping, grading, and assessment of other histologic features.

#### Immunohistochemistry

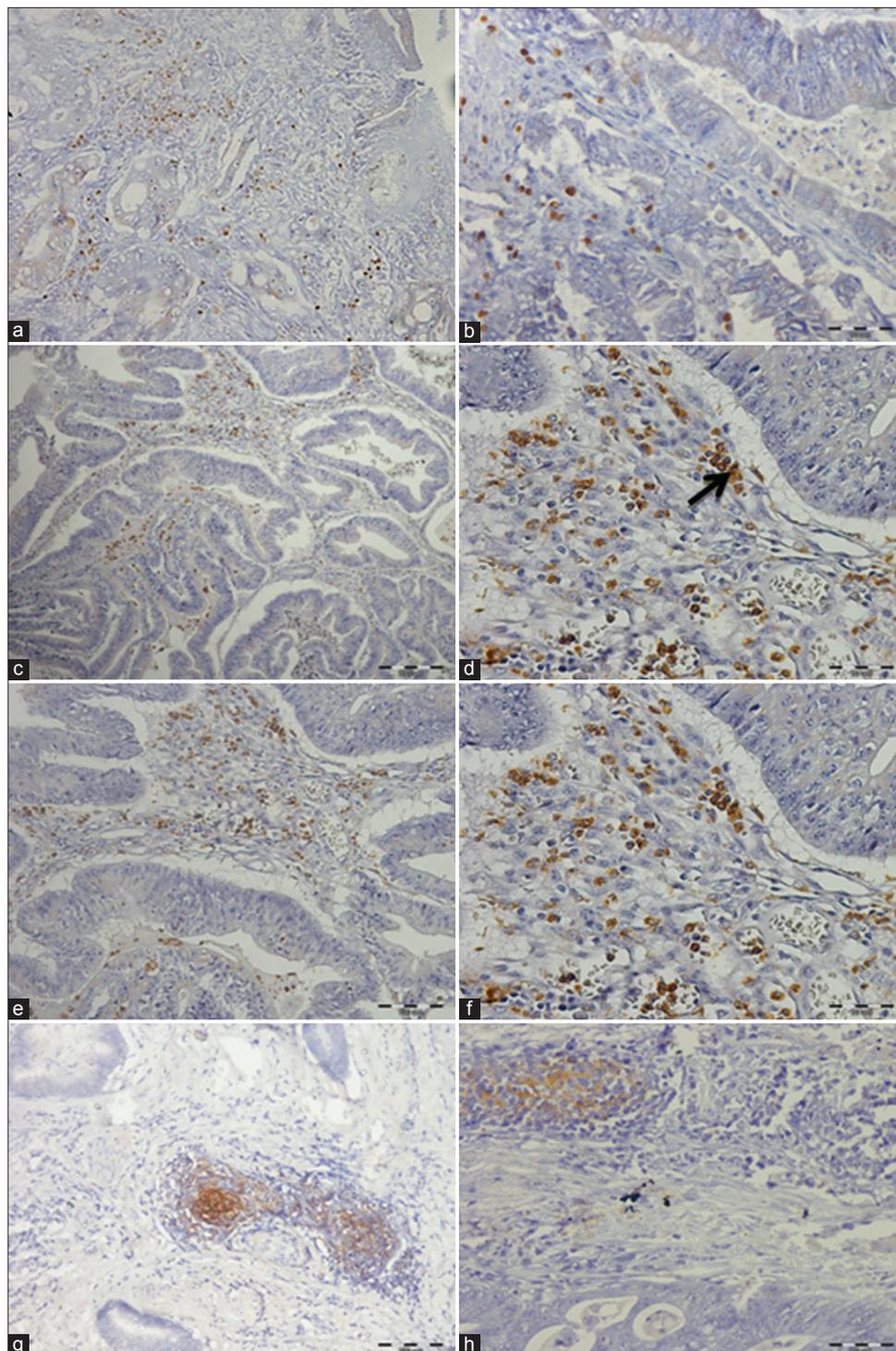
Staining was performed using streptavidin – biotin-peroxidase complex technique using the following antibodies, forkhead boxP3 (Foxp3) (clone 236A/E7), CD68 (clone KP1), CD8 (C8/144B), and CD21(clone FBCI). Sections of the tonsil were included as positive control for the four antibodies used (as recommended by the manufacture's protocols) [14]. Sections without primary antibodies served as negative control.

### Scoring of immunohistochemical staining

Slides for each antibody were separately examined under low and high power light microscopic examination for semiquantitative assessment of antibody – staining.

Positive CD68 was defined as cytoplasmic staining of the macrophage (Fig. 1e and f); percentage was evaluated using semiquantitative method described by Jakovic *et al.* ten randomly selected high power fields ( $\times 400$ ) were selected in the stroma infiltrated by inflammatory cells [15].

TIL including Treg and cytotoxic T lymphocytes subsets in CRC was determined immunohistochemical evaluations of Foxp3 and CD8, respectively, expression. Foxp3<sup>+</sup> TIL was counted in five randomly selected high power fields at ( $\times 400$ ) magnification in the stroma of cases according to method described by Takenaka *et al.* Positive Foxp3 was defined as nuclear or cytoplasmic staining (Fig. 1a and b), the percentage of (Foxp3) stained cells was scored according to four grades scoring systems. (0): Negative, (1+): 1–25%, (2): 26–50%, and (3): 51–100% [16].



**Fig. 1:** Expression of immune markers in CRC cases. – A x100, B x400 Foxp3 (brown nuclear) in the stroma. C x200, D x400: CD8 (positive T cells) the arrow indicate positive cells. E x100, F x400: CD68 (positive tumor associated macrophage in the stroma of CRC biopsy). G x100, H x200: TLS immunostained with an antibody against CD 21

Positive (CD8) was defined as membranous ± cytoplasmic staining (Fig. 1c and d). The percentage of CD8 stained cells was assessed according to scoring systems described by Chen *et al.* [17].

CD21<sup>+</sup> follicular dendritic cell (FDC) was defined as formed a tight network within the follicle as shown in Fig. 1g and h and was evaluated as either positive or negative staining according to the method described by Trajkovski *et al.* [10].

#### Follow-up

Cases were followed up for a period of 4 years. New events including tumor recurrence or metastasis were documented.

#### Statistical analysis

Data were fed to the computer and analyzed using IBM SPSS software package version 20 and Microsoft Office Excel 2010. Chi-square test was used to compare different groups. The significance of the obtained results was set at  $p < 0.05$ .

### RESULTS

In the studied 50 cases of CRC, the age of the patient ranged from 22 to 80 years with a mean of  $48.28 \pm 12.23$  years. Clinical and histologic data of the studied cases were summarized in Table 1.

#### Results of immunostaining

The mean for Foxp3, CD68, and CD8 was  $58.0 \pm 25.99$ ,  $68.30 \pm 13.61$ , and  $46.50 \pm 29.90$ , respectively, while positive CD21 was detected in 29/50 cases (58%).

Results of immunostaining and its correlation with other clinicopathological parameters were summarized in Table 2.

Foxp3 staining density was significantly higher in low grade ( $p = 0.005$ ) and low stage tumors ( $p = 0.021$ ).

The mean of CD8<sup>+</sup>ve CTC was significantly higher in males (mean  $60.0 \pm 29.83$ ,  $p = 0.042$ ), in the right-sided colon than other sites (mean  $54.38 \pm 32.60$ ,  $p = 0.042$ ), non-mucinous histological type ( $p = 0.036$ ), and low grade ( $p = 0.018$ ) tumors. The mean CD8 staining was higher in Stage I, II than Stage III but the result was statistically insignificant ( $p = 0.0616$ ).

CD68<sup>+</sup>ve macrophage percentage was significantly higher in the right-sided colon than other sites ( $p = 0.043$ ).

CD21 was significantly correlated with non-mucinous histological type ( $p = 0.021$ ) and low stage tumors ( $p = 0.004$ ).

#### Results of follow-up

During the period of follow-up, five cases developed metastasis. As regards the correlation with immunostaining, the overall survival was significantly higher in cases expressing high Foxp3 (mean = 55.80) in comparison to those with lower Foxp3 expression (mean = 34.10) ( $p < 0.001$ ) (Fig. 2a).

The overall survival also was higher in cases expressing high CD8 staining (mean = 53.89) than those with lower CD8 expression (mean = 35.0) ( $p = 0.014$ ) (Fig. 2b).

Overall survival was higher in cases with low expression of CD68 (mean = 34.0) than those with reveals high expression (32.70), but this result was statistically insignificant ( $p = 0.665$ ) (Fig. 2c).

Overall survival was higher (mean = 55.0) in cases shows CD21 positively stained FDC cells than cases did not show positive staining (mean = 41.29) ( $p = 0.043$ ) (Fig. 2d).

### DISCUSSION

TME has a key role in the breakdown of prison on tumor cells to interact with surrounding environment. CRC, identical to most other solid

**Table 1: Summary of clinical and histologic data of the studied cases**

Characteristic	n	%
Age		
≤50	29	58.0
>50	21	42.0
Sex		
Male	11	22.0
Female	39	78.0
Size		
≤5 cm	31	62.0
>5 cm	19	38.0
Tumor location		
Right colon	16	32.0
Left colon	17	34.0
Rectosigmoid	11	22.0
Rectum	6	12.0
Histologic type		
Non-mucinous	35	70.0
Mucinous	15	30.0
Grade		
Low grade	41	82.0
High grade	9	18.0
Stage		
I	9	18.0
II	21	42.0
III	20	40.0
Follow-up data		
Free	45	90.0
Relapse	5	10.0

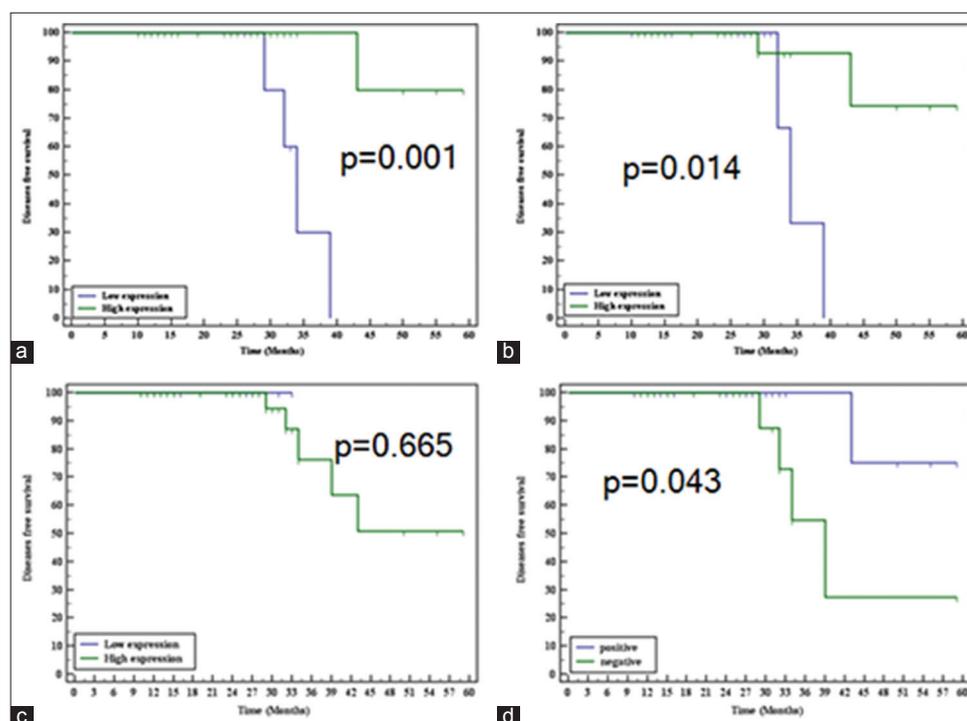
**Table 2: Results of immunostaining and its correlation with other clinicopathological parameters**

Parameter	Forkhead boxP3 density Mean±SD	CD8 density Mean±SD	CD68 density Mean±SD
Sex			
Male	60.0±28.37	60.0±29.83	95.91±17.15
Female	57.44±25.65	42.69±29.17	68.97±12.63
Significance (p)	0.602	0.042*	0.831
Size			
≤5	60.16±24.24	51.29±29.07	65.97±14.52
>5	54.47±28.96	36.68±30.36	72.11±11.34
Significance (p)	0.693	0.234	0.150
Site			
Right colon	65.31±24.46	54.38±32.60	72.50±14.26
Left colon	64.12±20.78	52.35±29.85	70.88±12.40
Rectosigmoid	44.55±30.12	24.09±21.89	65.0±12.45
Rectum	45.83±27.46	50.0±18.17	55.83±10.68
Significance (p)	0.064	0.042*	0.043*
Type			
Non-mucinous	61.0±23.85	50.29±28.26	69.29±13.29
Mucinous	51.0±30.13	37.67±32.73	66.0±14.54
Significance (p)	0.374	0.036*	0.465
Grade			
Low	63.41±23.44	51.59±29.74	68.54±13.98
High	33.33±23.58	23.33±17.85	67.22±12.53
Significance (p)	0.005*	0.018*	0.691
Stage			
I	64.44±27.55	49.05±28.36	66.67±12.25
II	65.24±19.20	48.75±28.19	68.10±15.20
III	47.50±29.0	35.56±37.62	69.25±13.01
Significance (p)	0.021*	0.616	0.862

Significant level\* if  $p \leq 0.05$

tumors has microenvironment rich in inflammatory cells including TIL and macrophage [6,10].

Transcription factor Foxp3 immunostaining was used to highlight T-regulatory cells. In our study, Foxp3 expression was significantly



**Fig. 2:** Kaplan–Meier survival curves for disease-free survival and its correlation with immunomarkers (a) Forkhead boxP3, (b) CD8, (c) CD68, and (d) CD21

higher in early localized CRC (with low grade and low stage) with higher overall survival and disease-free survival. Controversial data were reported about Foxp3 in the literature, Sun *et al.* [18], Ling *et al.* [19], and Ladoire *et al.* [20] reported significance correlation between Foxp3 and early tumor grade and stage as well as longer survival which confirms that high-density Treg cells is related to a good prognosis, while Grimmig *et al.* [21] and Michel *et al.* [22] reported higher Foxp3 expression in aggressive tumors. Kim *et al.* [23] reported no significant difference in overall survival and tumor-infiltrating Treg cells.

The discrepancy among these results might be attributed to sample size and experimental methods where we concentrated on the TIL in tumor center and excluded the lymphocytes infiltrating peritumoral area. In addition some authors reported immune suppressive effect for Treg cells by secreting immune suppressive cytokines such as interleukin 10 and transforming growth factor-B into microenvironment of tumor tissue, which leads to immunological destruction and tumor promotion. These cytokines were considered in tumors such as breast, hepatocellular carcinoma, and ovary as unfavorable prognostic markers. While in other tumors as in CRC, head and neck cancer associated with good prognosis and improvement survival by its ability to reduce proliferative cytokine in milieu.

Moreover, Kim *et al.* [23] reported that cancer cells generated Foxp3 are associated with bad prognosis while its production by infiltrating Treg cells is associated with favorable prognosis in CRC, this may explain the discrepancy between our results and other results where we studied the total density of Foxp3<sup>+</sup> ve Treg cells regardless of its origin.

Our study also included the assessment of macrophage density, at least two populations of macrophages with opposite immunological roles have been identified; as double edge swords, the M1 (classically activated) type with a proinflammatory antitumoral function and the M2 (alternatively activated) type with an immunosuppressive protumoral function [24,25]. It has been postulated that most of the tumor-associated macrophages belong to the M2 subtype [26]. For this reason, many studies attempted to trace tumor-associated macrophages using CD163 – a marker of M2 macrophages – with/without CD68.

However, a new hypothesis assumes that macrophages *in vivo* do not strictly follow the M1/M2 model, but rather tend to switch from one type to the other depending on the microenvironment activating signal they receive [26]. This being said, it is considered inappropriate to classify tumor-associated macrophages into either M1 or M2 type, but rather to consider them as a heterogeneous population playing different roles at different stages of tumor development [27-30]. Again, this may explain the discrepancy in the results of macrophages of different studies focusing on the prognostic role of macrophage in CRC including ours.

In the present work, immune histochemical identification was done using the most common and most commercially available routinely used pan-macrophage marker CD68 to include all macrophage populations in CRC, CD68 percentage was only significantly associated with site of tumor, with highest mean in right side of colon, although mean of CD68 was higher in Stage III, in metastatic, this difference was not statistically significant. Similar results were reported by Waniczek *et al.* [31] and Cui *et al.* [32] who concluded that increase intensity of macrophage is associated with unfavorable prognosis. Contradictory results were reported by Forsell *et al.* [33] and Li *et al.* [34] who found a significant positive correlation between intense infiltrations of CD68 and improved prognosis of CRC.

An additional explanation for this discrepancy may be also related to the heterogeneous distribution of macrophage in the different areas within same tumor, including tumor stroma, invasive borders, or peritumoral area as reported by Chaput *et al.* [35] who reported that of increase macrophage in tumor stroma of CRC is associated with poor prognosis and opposite finding with more macrophage at tumor in front which is associated with improve prognosis, in our study, we evaluated macrophage density in the stroma of CRC.

For CD8+CTC is reported that CTCs are having an antitumor effect through its capability to prohibit target cells on being exposed to tumor cell antigen/human leukocyte antigen 1 complex for which their T cell receptors are specific, leading to the destruction of tumor cells by apoptosis and this improving prognosis. Our results were concordant

with results reported by Deschoolmeester *et al.* [36], Ling *et al.* [19], Sideras *et al.* [37], and Shibutani *et al.* [38], regarding the antitumoral effect of CTC as it was significantly higher in early non-metastatic tumors.

The developments of tertiary lymphoid structure (TLS) in tumors have been depicted in several other neoplastic such as lung and breast malignant melanoma.

Lymphoid neogenesis that develops within the tumor resembles the aggregation in secondary lymphoid. Many studies describe the relationship between TLS and clinical outcome in tumors. TLS was detected microscopically by H and E staining and by immunohistochemistry in invasive CRC using CD21. TLS was found in (58%) of cases. There was a statistically significant correlation between the presence of TLS and non-mucinous histological type, lower stage tumors, and overall survival. Similar results were reported by Coppola [8] and Trajkovski *et al.* [10] who assumed that TLS is usually associated with lower grade and good prognosis in CRC and this may be explained by immune-supportive mechanisms attributed to those lymphoid aggregates, however, still contradicting findings were reported.

## CONCLUSION

To sum up, our results assumed that higher expression of Foxp3, CTC, and TLS is associated with favorable prognosis in CRC which reflects the synergistic effect of regulatory T cells and CTC in TME of CRC; however, the exact role for each one still needs more studies, while CD68 needs further work to precisely evaluate its exact role in CRC.

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## AUTHORS' CONTRIBUTIONS

The author announces that this work was done by the authors named in this article.

## CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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