The prognostic significance of tumor infiltrating CD8+ cytotoxic cells and FoxP3+T regulatory cells in colon cancer

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Abstract: Background: T-lymphocytic infitration may have a crucial role in the prognosis of colorectal cancer patients. Our aim was to assess the prognostic significance of the presence of lymphocytic infiltration with focusing on its subsets; CD8+ and FoxP3+. Tumor infiltrating lymphocytes (Tils) were quantified in 69 patients operated for stage II and stage III colon cancer. Patients with high Tils were further stained for CD8+and FoxP3+. Results Most of our patients (73.9%) had low LI, while 26.1 % had high LI. There was no significant association between any clinic-pathological feature and density of LI. After a median follow up of 56.5 months, the DFS and OS varied according to the density of LI. There was a trend towards better 3-year DFS and OS in patients with high LI (p=0.065) and (p=0.08) respectively. By immunophenotyping, CD8+ infiltration was more common than FoxP3+. Prominent CD8+ infiltration was associated with lower grade tumors (p=0.06), lower N stage (p=0.06) and favorable overall survival (p=0.07).

1. Introduction

The prognostic significance of tumor-infiltrating lymphocytes (TILs) has been a longstanding topic of debate. Many studies across a wide variety of human tumors have shown a significant association between the presence of TILs and patient survival [1–3].

However, it is important to distinguish between different types of T lymphocytes, because they may play different role in the tumor microenvironment. Most TILs have a CD3+ phenotype, and CD3+ TILs can be further subdivided into cytotoxic (CD8+) T cells, memory (CD45RO+) T cells, and regulatory (CD4+ CD25+) T cells [4,5]. Cytotoxic (CD8+) T cells are thought to have antitumor functions, therefore, a high density of CD8+ T cells has been shown to be associated with improved outcome [6,7].

The existing evidence suggests that regulatory T cells (Tregs) have the ability to inhibit host-versus tumor immunity in the tumor microenvironment via suppression of antitumor cytotoxic T cells [8,9].

The transcription factor forkhead box P3 (FoxP3) is a key intracellular molecule for Tregs development and function [10], which is considered to be the most specific Tregs marker so far. Under normal conditions, FoxP3+ Tregs are essential suppressors of antitumor responses and thus maintain immunological tolerance to host tissues [11]. High infiltration of FoxP3+ Tregs is expected to be associated with an unfavorable outcome. Thus, FoxP3+ Tregs are investigated as a potential prognostic factor and they may also represent a novel therapeutic target [12]. Recent evidence indicates that signaling of the T cell chemoattractant CCL5 can recruit Tregs to tumors and enhance their ability to kill CD8+ T cells, thereby providing a mechanism of immune escape [13].

Indeed, the poor prognostic effect ot high infiltration of FoxP3 has been reported in a wide range of localized or metastatic human carcinomas, including breast [14],ovarian [15],hepatocellular [16], lung [17], pancreatic [18], and gastric [19].

In colorectal cancer,the presence of tumor infiltrating lymphocytes (TIL), has proven to be a favorable independent prognostic factor [20]. However, high density of tumor-infiltrating FoxP3+ Tregs was shown to correlate with worse prognosis in some studies [21,22], while other studies identified a considerable association with favorable prognosis [23-26].

The favorable prognosis associated with high infiltration of FoxP3+ T cell has also been reported in other tumors as Hodgkin's and follicular lymphoma [27] and in head and- neck carcinomas [28].

Thus, a deep understanding of the prognostic role of FoxP3 is warranted.

The aim of our work is to assess the pattern and prognostic significance of tumor-infiltrating lymphocytes with focusing on its subtypes (CD8+ and Foxp3+ cells) in stage II and III colon cancer

2. Methods

Ethics Statement

The study was approved by our local ethics committee

Patients

Formalin-fixed paraffin-embedded specimens of 87 patients, with surgically resected TNM stage II and stage III primary colon cancer were collected. These patients were treated with curative intent in our institution between 2010 and 2014.We retrospectively reviewed their medical records and obtained all the clinic-pathological characteristics, treatment Information and patient follow up data. We excluded 10 patients with unavailable follow up data and 8 patients with bad tumor fixation. Finally, 69 patients were eligible for our study. The median duration of follow up was 56.5 months.

Immunohistochemistry

Formalin-fixed paraffin-embedded blocks from the patients were studied for histologic subtype, grade, pathological stage and density of tumor infiltrating lymphocytes in both epithelial and stromal compartments on H & E stained sections. TILs were scored into low (<50%) and High (>50%) of tumor sections.Immuno-histochemical studies using anti CD8 and Fox P3 antibodies applied on 3-4μ thick sections mounted on positively charged slides. Sections were boiled in citrate Buffer (PH6)for 15 minutes, then incubated with primary antibodies at following data For CD8+and FoxP3+ cases, positively-stained cells were counted within the tumor at 20x magnification. Density measurements were recorded as the number of positive cells per tumor per 0.28 mm2 surface area). Then scores were classified into low and high (prominent). Immunohistochemistry for CD8 and Fox P3 was done only in cases of high LI (Figure1).

Table 1. Association of clinicopathological Characteristics with density of Lymphocytic Infiltration

	LI high (n = 18)	LI low (n = 51)	p
Age			
Mean ± SD	47.9 ± 14.5	48.7±18.3	0.38
Median	44	46	
Sex			
Males	10 (55.5%)	31(60.8%)	0.89
Females	8 (44.5%)	20(39.2%)	
Site of tumor			
Right	5 (27.8%)	16 (31.4%)	0.67
Left	13 (72.2%)	35 (68.6%)	
Tumor size			
<5cm	6 (33.3%)	18 (35.3%)	0.74
≥5cm	12 (66.7%)	33 (64.7%)	
Grade			
Well differentiated	2 (11.1%)	4 (7.8%)	
Moderately differentiated	12 (66.7%)	30 (58.8%)	0.65
Poorly differentiated	3 (16.7%)	12 (23.6%)	
Undifferentiated	1 (5.5%)	5 (9.8%)	
T			
1	3 (16.7%)	6 (11.8%)	
2	4 (22.2%)	10 (19.6%)	0.73
3	10 (55.6%)	31 (60.8%)	
4	1 (5.5%)	4 (7.8%)	
N			
Positive	6 (33.3%)	19 (37.5%)	0.84
Negative	12 (66.7%)	32 (62.7%)	
LVI			
Negative	10 (55.6%)	38 (74.5%)	0.1
Positive	8 (44.4%)	13 (25.5%)	
Obstruction and perforation			
No obsruction or perforation	17 (94.5%)	47 (92.1%)	0.98
Obstruction and/or perforation	1 (5.5%)	4 (7.8%)	
Relapse			
No	13(72.2%)	33 (64.7%)	0.89
Yes	5 (27.8%)	18 (35.3%)	

Statistical Analysis

Statistical analysis of the dataData were fed to the computer and analyzed using IBM SPSS software packageversion 20.0. (Armonk, NY: IBM Corp). The Kolmogorov- Smirnov test was used to verify the normality of distribution of variables, Comparisons between groups for categorical variables were assessed using Chi-square test (Fisher or Monte Carlo). Disease-free survival (DFS) was defined as the time from date of surgery until death from any or the date of first locoregional or distant recurrence. Overall survival (OS) was calculated from the date of surgery until death from any cause or was censored at last follow-up. Both DFS and OS were estimated by using the Kaplan-Meier methods. For the univariate analysis, Kaplan-Meier method with the log rank test was used. Significance of the obtained results was judged at the 5% level.

3. Results

The baseline clinic-pathological characteristics of the eligible patients, stratified by the density of lymphocytic infiltration, are listed in Table 1.

Most of our patients (73.9%) had low LI, while 26.1 % had high LI. There was no significant association between any clinic-pathological feature and density of LI.

After a median follow up of 56.5 months, the DFS and OS varied according to the density of LI.

There was a trend towards better 3-year DFS in patients with high LI compared to low LI (85% versus79 %) (p=0.065) (Figure 2).

The 3-year OS was also numerically better in high LI compared to low LI (96% vs 90 %) (p=0.08) (Figure3).

Immunophenotyping:.

Tumors with high LI were further stained with CD8+and FoxP3+.Prominent staining for CD8+ was observed in 88.9% of the cases (either alone or associated with high FoxP3+), while prominent FoxP3+ was noticed in 61% of tumors either alone or with high CD8+.

Association of CD8+ and FoxP3+ densities with different clinic-pathological parameters are presented in Table 2. No significant correlations were observed between the density of CD8+ or FoxP3+ and any of the clinic-pathological features. However, there was a tendency for lower grade in tumors with prominent CD8+ (p= 0.06) and FoxP3+ (p=0.059) and lower N stage in tumors with prominent CD8+ (p=0.06).

In univariate analysis, Older age, lower grade and stage were significantly associated with better overall- survival. Prominent CD8+ and not FoxP3 was a predictor of more favorable overall survival (p=0.07) (Table3).

**Table 2. Association of clinicopathological Characteristics with density of CD8+ and FoxP3+.**

	High CD8+ T cell density		High FoxP3+ T Cell Density	
	N (%)	P	N (%)	p
Age				
< 40	6 (37.5%)	0.64	4 (36.4%)	0.65
≥ 40	10 (62.5%)		7 (63.6%)	
Sex				
Males	9 (56.2%)	0.98	6 (54.5%)	0.92
Females	7 (43.8%)		5 (45.5%)	
Site of tumor				
Right colon	5 (31.2%)	0.09	4 (36.4%)	0.65
Left colon	11 (68.8%)		7 (63.6%)	
Tumor size				
< 5 cm	6 (37.5%)	0.73	4 (36.4%)	0.8
≥ 5 cm	10 (62.5%)		7 (63.6%)	
Grade				
Well differentiated	2 (12.5%)		1 (9.1%)	
Moderately differentiated	11 (68.7%)	0.06	8 (72.7%)	0.059
Poorly differentiated	2 (12.5%)		2 (18.2%)	
Undifferentiated	1 (6.25%)		0	
T				
1	3 (18.7%)		2 (18.2%)	
2	4 (25%)	0.65	3 (27.3%)	0.82
3	9 (56.2%)		5 (45.4%)	
4	0 (0)		1 (9.1%)	
N				
Positive	4 (25%)	0.06	5 (45.5%%)	0.1
Negative	12 (75%)		6 (54.5%%)	
LVI				
Negative	9 (56.2%)	0.43	7 (63.6%)	0.65
Positive	7 (43.8%)		(36.6%)	

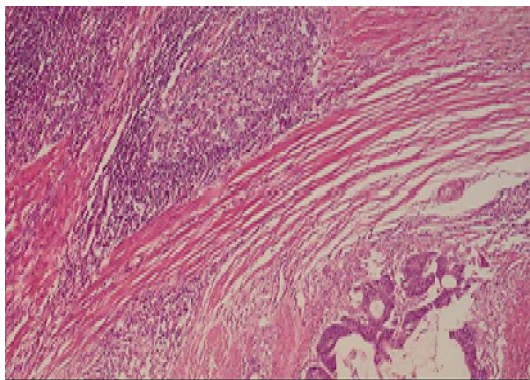**Table 3. Univariate analysis for the parameters affecting overall survival (n= 69)**

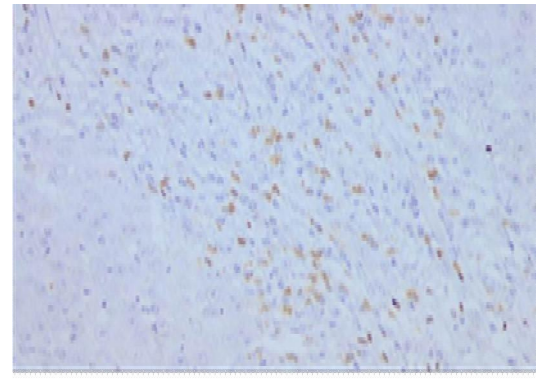|	HR	95% CI		P
		LL	UL	
Age	0.9	0.9	0.9	0.04
Sex				
Males vs Females	0.85	0.5	1.2	0.34
Grade				
Well or Modertely vs Poor or Undifferentiated	1.34	1.1	2.1	0.02
Stage	2.6	2.3	4.3	0.04
II				
III				
Tumor site				
Right vs Left	1.4	1.01	2.3	0.73
CD8+ T-cell Density				
High vs Low	0.78	0.69	0.94	0.07
FoxP3+ T-cell Density				
High vs Low	0.69	0.45	1.12	0.183
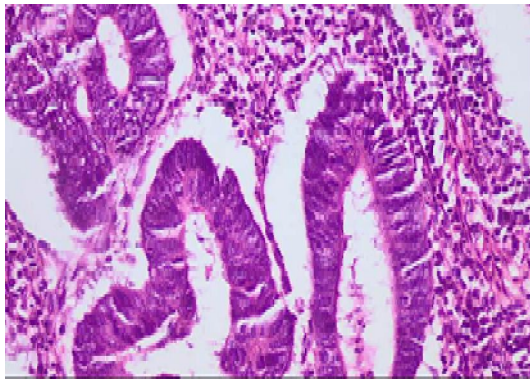
(A)
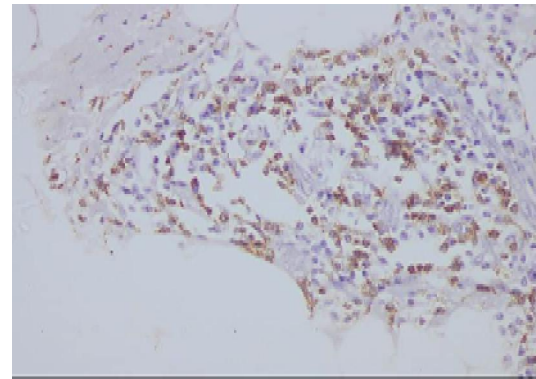
(C)

(B)

(D)

Figure (1) Examples of the presence of lymphocytic infiltration (LI) and its subsets (CD8+, FoxP3+) in the primary tumors

(A) H & E stained section showing high intratumoral lymphocytes (x400)

(B) H & E stained section showing high peritumoral lymphocytes (x100)

(C) High intratumoral CD8 cytoplasmic immunoreactivity (ABC x400)

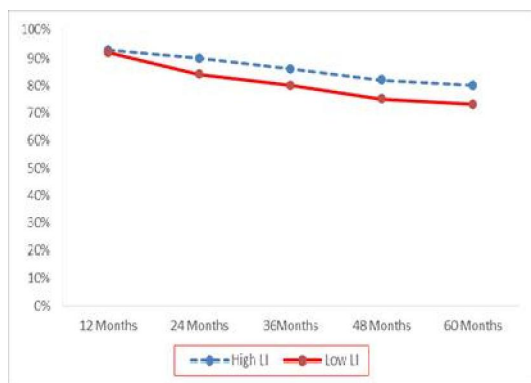(D) High intratumoral FOXP3 nuclear positivity (ABC x400)

Figure (2) Disease free survival according to density of Lymphocytic infiltration
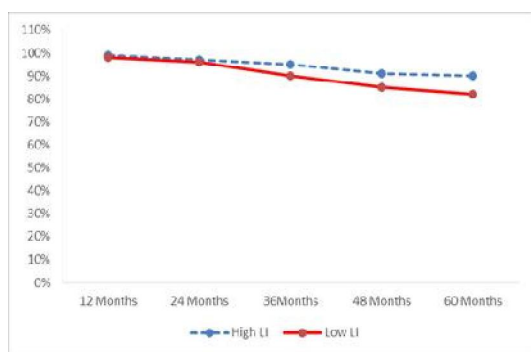

Figure (3) Overall survival according to density of lymphocytic infiltration

4. Discussion

The prognosis of colorectal cancer used to be predicted by the classical clinic-pathological features as TNM stage, lymphovascular invasion and age [29], but recently host-defense mechanisms emerged as an important factor in p redicting the outcome of patients with colorectal cancer [30,31].

The prognostic role tumo–infitrating lymphocytes have been evaluated in colorectal cancer, but most studies include patients with colon and rectal cancer, only few studies have focused on colon cancer [32,33].

Moreover, TIls have several subsets, each has a specific contribution in controlling the local -adaptive immune response and there is also a complex interaction among theses subsets. [34]

The objective of our study is to assess the pattern of TIls and its prognostic singnificance, with specific emphasis on its subtypes; CD8+ and Foxp3+ in patients with stage II and stage III colon cancer.

In our study we quantified TIls in the stroma and epithelium of the tumor together.

Most of our patients (74%) had low LI. Patients with high LI (26%) showed a tendency for better DFS and OS. The 3-year DFS and OS was 85% and 96% in patients with high LI, compared to 79% and 90 % in patients with low LI, respectively.

This is in accordance to Emile et al [35]. who reported a higher DFS and OS in patients with high LI compared to low lI.

Pages et al [36]. also, found that high levels of TIls are associated with less advanced stage and longer overall survival.

In our study patients with high LI were further analyzed and stained for CD8+ and FoxP3+.

CD8+ density was higher than FoxP3+. The prescence of high infiltration of CD8+was associated with lower grade (p=0.06) and lower N stage (p=0.06).In univariate analysis, we found that CD8+ was associated with a tendency for favorable overall survival (p=0.07).

According to our results, TIls especially its subset CD8 densities were shown to add prognostic information to the classical clinico-pathological features.

Our study has some limitations. First being retrospective. Second including small sample of patients.

Conflict of interest

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Author contribution:

Azza Darwish, Haitham Fayed and Dina Abdallah contributed equally to this work.

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