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ORIGINAL ARTICLE

The prognostic value of FOXP3$^+$ T regulatory cells in colorectal cancer

Catalin Vlad[1], Paul Kubelac[2], Bogdan Fetica[1], Diana Vlad[3], Alexandru Irimie[1], Patriciu Achimas-Cadariu[1]

[1]Department of Surgical and Gynecological Oncology, Ion Chiricuta Institute of Oncology, Cluj-Napoca; [2]Department of Oncology, Faculty of Medicine, Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca; [3]Department of Otorhinolaryngology, CFR Hospital Cluj-Napoca, Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania

Summary

Purpose: *The evaluation of CD3$^+$ T-cell density is believed to have a higher prognostic value than the conventionally used TMN stage in colorectal cancer (CRC), but the role of regulatory T lymphocytes (Treg) is still debated. Our study determined the prognostic value of forkhead box P3 nuclear transcription factor (FOXP3) positive Treg and CD3$^+$ T-cells in the invasive margin of CRC compared with other known prognostic factors.*

Methods: *The prognostic factors analysed in 42 patients with CRC stage II (N=13) and III (N=29), were age, tumor location, TNM stage, histological grade, vascular, lymphatic and perineural invasion. CD3$^+$ T-cells and FOXP3$^+$ Treg density was evaluated by immunohistochemistry.*

Results: *The median CD3$^+$ T-cells and FOXP3$^+$ Treg density was 438.93/mm^2 and 162.25/mm^2, respectively. Patients with high FOXP3$^+$ Treg density showed improved 5-year survival rate of 89.41%, compared with 64.6% of those with low density (p=0.024).*

Conclusions: *Increased CD3$^+$ T-cells and FOXP3$^+$ Treg density is associated with improved survival, but only the latter proved to be an independent prognostic factor. FOXP3$^+$ Treg infiltrate may play an important prognostic role, which, in combination with other predictive factors, could lead to the development of specific treatment regimens.*

Key words: *colorectal cancer, forkhead transcription factors, prognosis, tumor infiltrating lymphocytes*

Introduction

CRC progression depends on the interaction between tumor aggression factors and host immune response, involving the interactions of several cell types and cellular products, belonging to the immune system [1,2].

The presence of tumor infiltrating lymphocytes (TIL), has proven to be a favorable independent prognostic factor in CRC, hence the evaluation of CD3$^+$ T-cells density would have a higher prognostic value than the conventionally used TMN stage [3]. CD3$^+$ T-cells subpopulations involved in this phenomenon have been characterized in ovarian cancer, with an emphasis on cytotoxic T lymphocytes (CD8$^+$) and regulatory T cells (CD4+, CD25$^+$, FOXP3$^+$) [4,5].

Increased tumor infiltration by CD8$^+$ and CD45RO$^+$ T-cells has been shown to be correlated with a favorable prognosis in CRC, independent of the microsatellite instability status [6-8]. High intratumoral CD8$^+$ and CD45RO$^+$ densities are associated with low malignant invasive potential, early tumor stages and improved survival [9,10].

The role of Treg in the pathology of CRC is still debated. FOXP3 is particularly expressed on Treg, playing an important role in the development and function of these cells [11,12]. FOXP3$^+$ Treg suppress immune activation, maintaining

Correspondence to: Catalin Vlad, MD, PhD. Ion Chiricuta Institute of Oncology, Cluj-Napoca, Romania. 34-36 Republicii Street, 400015 Cluj-Napoca, Cluj, Romania. Tel: +40 740 256 076, E-mail: catalinvlad@yahoo.it
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immune system homeostasis and tolerance to self-antigens. Thus, they may have an inhibitory effect on the anti-tumoral immune response by reducing the activity of cytotoxic T cells. Considering this, patients with increased FOXP3+ Treg were initially thought to correlate with poor prognosis, however, recent studies show these patients, controversially, show better prognosis [13].

In this regard, we performed a retrospective study in which the main purpose was to determine the prognostic value of FOXP3+ Treg density in the invasive margin compared with CD3+ T-cells density and other known clinical and pathological prognostic factors.

Methods

Forty two patients diagnosed and treated for CRC at the Ion Chiricuta Institute of Oncology between 2001 and 2010 were included in the present study. The inclusion criteria were: patients with stage II and III CRC (according to the 7th edition of the UICC/AJCC) [14], who underwent primary surgical treatment, and presented a moderate or a high degree of TIL on histopathological examination.

Among the prognostic factors analyzed were age, tumor location, TNM stage, vascular invasion, lymphatic invasion, perineural invasion, and tumor histological grade. Tumor location was classified as proximal or distal, depending on the position from the splenic flexure. Histological grades were classified as low (well and moderately well differentiated tumors) and high (poorly differentiated and undifferentiated tumors).

CD3+ T-cells and FOXP3+ Treg density, expressed as the number of positive cells/mm^2, was categorized as high or low in relation to the median value. All patients included in the study deceased due to CRC, so the cancer-specific survival could be calculated.

The patient follow-up time was the interval between the date of surgery and the date of the latest information (death or completion of the study). The date of completion of the study was August 2012.

Tissue microarray technology was used for immunohistochemical assays and samples were analyzed by the same pathologist. The Leica BOND-III (Nussloch, Germany) instrument was used for dual staining of 5 μm thick sections.

The first primary antibody used was the anti-FOXP3 antibody (ab20034, Abcam, Cambridge, UK), at a dilution of 1:100, and the secondary antibody used was the Bond Polymer Refine Detection kit (DS9800, Leica Biosystems (Nussloch, Germany). The second primary antibody used was the anti-CD3 antibody (A0452, Dako, Glostrup, Denmark), at a dilution of 1:100, and the second secondary antibody used was the Bond Polymer Refine Red Detection kit (DS9390, Leica Biosystems (Nussloch, Germany) (Figure 1).

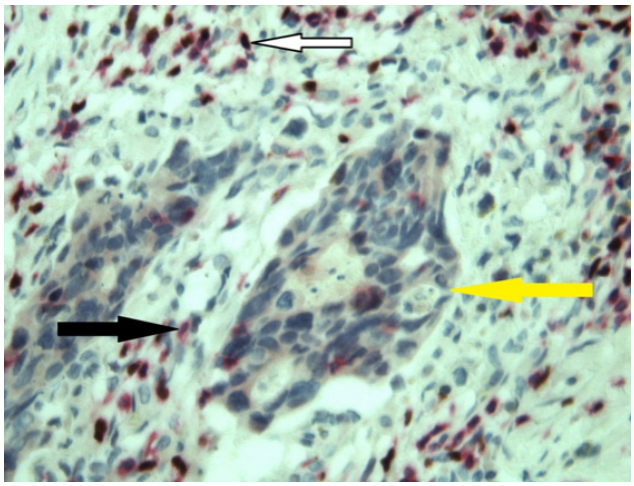

Figure 1. Immunohistochemical staining against FOXP3 and CD3 antigen (×40). The black arrow points red intracytoplasmic staining (CD3+) without brown nuclear staining (FOXP3-) T lymphocyte. The white arrow points red intracytoplasmic staining (CD3+) with brown nuclear staining (FOXP3+) regulatory T lymphocyte. The yellow arrow points islet of tumor cells.

Statistics

Data was analysed using descriptive and inferential statistics. For each set of values, appropriate statistical tests were applied (chi square, Fisher's exact test). Survival data was analysed using Kaplan-Meier method with log-rank test, and Cox proportional hazards model. For all tests, a p value < 0.05 was used as the threshold for statistical significance.

Results

The patient median age was 58 years (range 24-79). Twenty nine patients (69.1%) had stage III tumors and only 13 (30.9%) had stage II tumors. Most patients (76.2%) had low grade tumors. Venous invasion was present in 36 (85.7%) patients, lymphatic invasion in 20 (47.6%), and perineural invasion in 33 (78.6%).

The median CD3+ T-cells and FOXP3+ Treg density was 438.93/mm^2 and 162.25/mm^2, respectively. The complete clinical and pathological characteristics of the patients are shown in Table 1.

The 5-year overall survival was 75.6% (95% CI 0.626-0.911). The correlation between high CD3+ T-cells, FOXP3+ Treg densities and other clinicopathological characteristics are presented in Table 2. We observed that a high CD3+T-cells and FOXP3+ Treg density was associated with stage II disease. We also found that a high CD3+ T-cells density was correlated with low grade tumors, distal localization and no lymph node in-

Table 1. Patient clinical and pathological characteristics

Characteristics	*N (%)*	*Characteristics*	*N (%)*
Age, years		Venous invasion	
≤58	22 (52.4)	Yes	36 (85.7)
>58	20 (47.6)	No	6 (14.3)
Location		Lymphatic invasion	
Proximal	10 (23.8)	Yes	20 (47.6)
Distal	32 (76.2)	No	22 (52.4)
T stage		Perineural invasion	
T1,T2	9 (21.4)	Yes	33 (78.6)
T3,T4	33 (78.6)	No	9 (21.4)
N stage		CD3+ T-cells density	
pN0	13 (30.9)	High	22 (52.4)
pN+	29 (69.1)	Low	20 (47.6)
TNM stage		FOXP3+ Treg density	
II	13 (30.9)	High	21 (50)
III	29 (69.1)	Low	21 (50)
Grade		Intratumoral lyphocytic infiltrate	
Low	32 (76.2)	Moderate	15 (35.7)
High	10 (23.8)	High	27 (64.3)

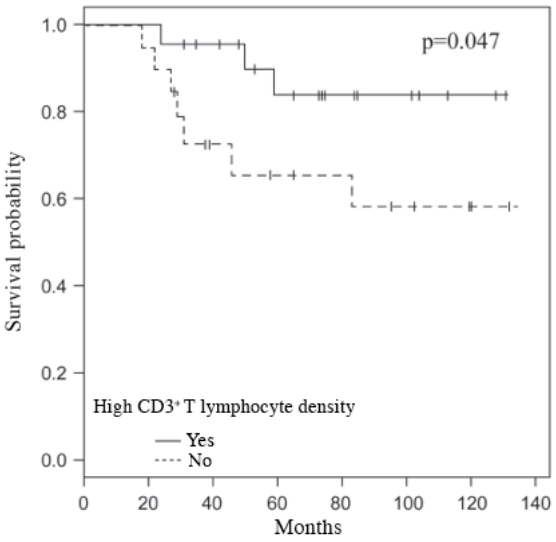

Figure 2. Overall survival depending on CD3+ T lymphocyte density.

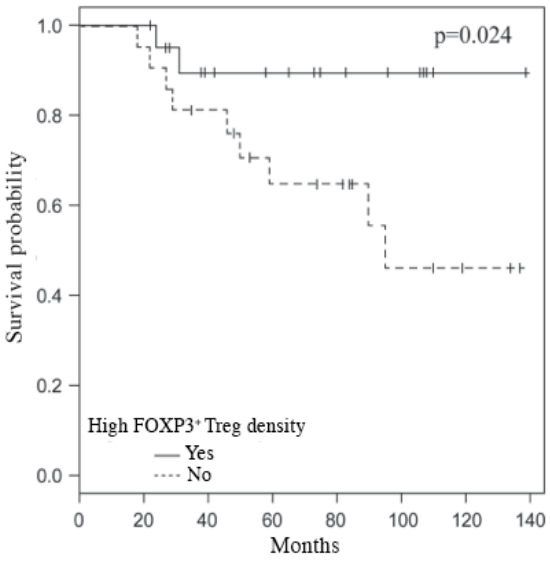

Figure 3. Overall survival depending on FOXP3+Treg density.

volvement.

In univariate analysis the 5-year overall survival rate for several variables was calculated, and presented in Table 3. A high CD3+ T-cells and FOXP3+ Treg density, age, TNM stage, lymphatic, venous and perineural invasion were found to have prognostic significance in colorectal cancer. Figures 2 and 3 illustrate the overall survival rate depending on CD3+ T-cells and FOXP3+ Treg density, respectively. The 5-year survival rate was higher in patients with high CD3+ T-cells density (p=0.047), as it was in patients with high FOXP3+ Treg density (p=0.024).

In multivariate analysis TNM stage, lymphatic invasion and FOXP3+ Treg density were shown to be independent prognostic factors, as shown in Table 4.

Discussion

CRC is one of the most common malignancies that occur in developed countries [15], and surgical resection is still the main treatment of choice. For stages II and III patients who are at high risk of relapse, adjuvant chemoradiotherapy is recommended after optimal surgery [16-18]. Despite the introduction of new chemotherapeutic agents which improved the prognosis of CRC, the outcome for most patients remains relatively poor [15,17].

New approaches focus on the role of host immunity in the development of CRC [9]. Treg lymphocytes can suppress immune responses to peripheral tumor antigens, and therefore contribute to tumor progression and dissemination, as shown in various cancers such as melanoma [19], breast [20] and ovarian cancer [5], hepatocellular carcinoma [21] and pancreatic cancer [22].

Table 2. Correlation between high CD3+ T-cells and high FOXP3+ Treg densities and other clinicopathological factors

Clinicopathological factors	*High CD3+ T-cells density*		*High FOXP3+ Treg density*	
	HR	*p value*	*HR*	*p value*
TNM stage		0.005		0.039
II	1.34		1.28	
III	0.82		0.96	
T stage		0.269		1
T1, T2	0.61		0.84	
T3, T4	1.04		1.06	
Localisation		0.03		1
Proximal	0.67		1.22	
Distal	1.28		1.22	
Grade		0.03		0.469
Low	1.24		0.94	
High	0.67		1.11	
Venous invasion		0.665		0.663
Yes	0.8		0.86	
No	1.14		1.34	
Lymphatic invasion		0.361		1
Yes	1.04		1.06	
No	1.42		1.06	
Perineural invasion		1		0.665
Yes	0.96		0.82	
No	1.16		1.26	
N stage		0.005		0.739
N0	1.26		1.42	
N+	0.77		1.06	
Age , years		0.361		0.537
≤58	1.21		1	
>58	1		1.24	

Table 3. Univariate analysis of the 5-year survival rate

Variables		*5-year OS (%)*	*p value*
Age, years	≤58	88.67	0.025
	>58	63.51	
TNM stage	II	92.31	0.041
	III	67.31	
Lymphatic invasion	No	86.12	0.019
	Yes	56.7	
Perineural invasion	No	100	0.024
	Yes	67.35	
CD3+ T-cells density at invasive margin (median 438.93/mm²)	High	83.85	0.047
	Low	62.89	
FOXP3+ Treg density at invasive margin (median 162.25/mm²)	High	89.41	0.024
	Low	64.6	
CD3+ T-cells/FOXP3+ Treg	High	64.07	0.099
	Low	89.41	

Table 4. Multivariate analysis of prognostic factors

Variables	*HR*	*p value*	*95% CI*
Age	1.44	0.27	0.84 - 2.04
TNM stage	2.29	0.013	1.25 - 3.81
Lymphatic invasion	1.78	0.037	1.21 - 2.63
Perineural invasion	2.06	0.48	0.81 - 4.23
CD3+ T-cells density at invasive margin (median 438.93/mm²)	0.94	0.86	0.48 - 1.82
FOXP3+Treg density at invasive margin (median 162.25/mm²)	0.62	0.019	0.47 - 0.91

In regard to CRC, data is still controversial, some studies demonstrating that increased Treg tumoral infiltration is associated paradoxically with a favorable prognosis [13].

In accordance with previous reports [8,13,23], our results show that a high CD3+ T-cell density at the invasive margin is associated with improved survival, early stage, absence of node invasion, low grade tumors and distal tumor location. The density of CD3+ T-cells at the invasive margin could be characterized as a good indicator for an effective immune surveillance.

In our study, high density FOXP3+ T lymphocytes infiltrate has been proven to be an independent favorable prognostic factor. The CD3+ T-cells/FOXP3+ Treg ratio in the present research was not shown to have a statistically significant prognostic value. Patients with stage II CRC showed greater FOXP3+ Treg infiltrate than those with stage III CRC, suggesting a possible protective role against dissemination of malignant cells [24]. Univariate analysis of survival confirmed the poor prognosis associated with the presence of con-

ventional pathological prognostic factors, such as TNM stage, lymphatic invasion and perineural invasion, however at multivariate analysis, only tumor stage and lymphatic invasion proved to be independent factors, limited by the relatively small number of cases.

The improved prognosis associated with increased intratumoral lymphocytic infiltrate reveals an effective antitumor immune response [3,10], but, at the same time it could indicate an increased antigenic tumor phenotype that did not acquire the ability to evade the host immune system. A recent study suggests that FOXP3+ Treg density evaluation in tumor tissue and normal colorectal tissue, in association with vascular and perineural invasion, can increase the prognostic accuracy of patients with early stage CRC [25].

Previously published data regarding the effect of FOXP3+ Treg in the pathology of CRC are rather inconclusive regarding their prognostic relevance [24-26], and the lack of consistent results could be related to differences in the studied population sizes [13,25].

Another aspect currently studied is the distinctive role that FOXP3+ Treg density could have, depending on its intratumoral location [27], which may contribute to the lack of prognostic significance of FOXP3+ Treg or CD3+ T-cells infiltrating the tumor stroma, whereas the decreased intraepithelial CD3+ T-cells/FOXP3+ Treg ratio and low CD3+ T-cells density were independently associated with shorter disease-free survival.

Consistent with the initial hypothesis that FOXP3+ Treg inhibit the antitumor immune response, in some studies FOXP3+ Treg were associated with poor prognosis. However, recent studies have found that FOXP3+ Treg lymphocytes tumor infiltrate is associated with favorable outcomes in CRC. To explain this phenomenon several hypotheses have been emphasized: the presence of Treg is an indirect sign of an existing effective antitumor response [28]; there are several subtypes of Treg lymphocytes with different functions (conditioned by the unique colonic microbiological environment), which, under specific conditions, can activate a Th-17-dependent inflammatory response against endoluminal bacteria, which in turn, promotes a strong immuno-suppressive effect (and therefore a pro-tumoral outcome), led by Treg lymphocytes [29]. Given the lack of prognostic value of the CD3+ T-cells/FOXP3+ Treg ratio in our study, we tend to agree with the latter hypothesis. The biological properties of FOXP3+ Treg cells in cancer progression appear to be influenced by the tumor microenvironment in which they activate, but further studies are necessary to investigate this aspect.

In conclusion, the increased CD3+ T-cells and FOXP3+ Treg density in the invasive margin is associated with improved survival in stage II and III CRC, but only the latter proved to be also an independent prognostic factor. FOXP3+ Treg infiltrate may play an important prognostic role for patients with CRC, which, in combination with other predictive factors, could lead to the development of specific treatment regimens. Regarding the FOXP3+ Treg role as a potential therapeutic target, we believe that caution is required in both attempts of stimulation or inhibition of their activity. Future studies are necessary to enlighten their function.

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