

# Tertiary Lymphoid Structures in Colorectal Cancers and Their Prognostic Value

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## Abstract

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**BACKGROUND:** Tumor-infiltrating lymphocytes (TIL) in tumour stroma are considered to be involved in the elimination of malignant cells and prevention of metastasis formation. TIL consist of T lymphocytes including cytotoxic lymphocytes that are a constituent part of the effector mechanism of anti-tumour immunity and B lymphocytes that can form tertiary lymphoid structures (TLS). TLS has been described in several solid tumours and colorectal carcinoma (CRC), and the influence on the local and systemic anti-cancer response.

**AIM:** This study aimed to quantify the presence of TLS in CRC patients and to determine their role in tumour progression.

**PATIENTS AND METHODS:** The study included 103 patients with CRC who underwent surgery at the University Clinic of Digestive Surgery in Skopje, whose operative material was analysed at the Institute of Pathology, Medical Faculty in Skopje. The density of TLS was determined and correlated with the neoplasm status of local growth (T), positive lymph nodes, lymphatic invasion, and stage of the disease and tumour grade.

**RESULTS:** The density of TLS was significantly higher in patients with higher stage, lower T status, and negative lymph nodes, in patients with no lymphatic invasion and with better-differentiated tumours.

**CONCLUSION:** The density of TLS plays an important role in controlling the tumour growth, and it can be a parameter for neoplasm progression in CRC patients. The density of TLS influences the control of tumour progression.

## Introduction

Tumour stroma (cancer-associated stroma), as opposed to normal-tissue stroma, is a suitable environment for spreading of cancer and plays a key role in the growth and development of malignant neoplasms [1] [2]. It is considered that cellular-stromal interactions in malignant tumours play a significant role in the process of their progression. During this process, tumour cells are influenced by signals

coming from stromal, endothelial, inflammatory and immune cells [3]. Inflammatory cells in tumour stroma have a double role; they are involved in tumour progression, and tumour-infiltrating lymphocytes or so-called tumour-associated lymphocytes (TAL), which are also present in the stroma of solid malignant tumours, are involved in the elimination of malignant cells and prevention of metastasis [4] [5]. TALs consist of T lymphocytes including cytotoxic lymphocytes, which are a constituent part of the effector mechanism of anti-tumour- immunity, and B-

lymphocytes that can form tertiary lymphoid structures [5] [7].

Formation of tertiary lymphoid structures (follicles) (TLS) has been described in several solid tumours and colorectal carcinoma (CRC) [5]. They are transient accumulations of lymphoid cells that develop in non-lymphoid tissue in case of chronic inflammation of this tissue. They are built identical to lymph follicles of lymphoid organs, that is, lymph nodes. They contain B cell zone, which can form germinal centres, T cell zone, mature dendritic cells and high endothelial venules [7].

It is assumed that, in addition to other inflammatory cells, B lymphocytes play a significant role in the formation of inflammatory infiltrate during the onset of colorectal cancer (CRC) [5] [8], and TLS detected in tumours influence the local and systemic anti-cancer response [8].

This study aimed to quantify the presence of TLS in CRC patients and to determine their role in tumour progression.

## Material and Methods

The analysed patients in this study have clearly defined a cohort of patients with CRC, whose a tumour infiltrating lymphocytes along with B CD20+ cells were previously analysed and those results have already been published [9].

A total of 103 patients with CRC were included in the study. Sixty-eight (66.2%) were men and 35 (33.98%) women, with a mean age of  $64.57 \pm 11.5$  years; they all underwent surgery at the University Clinic of Digestive Surgery in the period from 2013 to 2017. The operative material was analysed at the Institute of Pathology in Skopje, where the diagnosis was confirmed, and the pTNM stage was determined.

In this study we analyzed the elements of the TNM classification: T status (local growth of the tumor), the presence of positive lymph nodes (LN), the disease stage (according to TNM classification 2010) [10] as well as the grade of tumor differentiation (G), and the density of TLS.

TLS was detected microscopically on routine prepared slides. Areas of an invasive tumour front with a high density of TLS were chosen, and additional sections for immunohistochemistry were made.

The TLS was defined by immunohistochemical staining with antibodies against CD4 (Dako Monoclonal Mouse Anti-Human CD4, Clone 4B12); CD8 (Dako Monoclonal Mouse Anti-Human CD8, Clone C8/144B); CD20 (Dako

Monoclonal Mouse Anti-Human CD20, Clone L26); and CD21 (Dako Monoclonal Mouse Anti-Human CD21, Clone 1F8) with a standard procedure using Immunoperoxidase LSAB + system.

The number of TLS was counted in 10 consecutive low-power fields (10 h 4) in the invasive front of the neoplasm. They were quantified as: 0 – no TLS found in 10 low-power field; + - 1 to 5 TLS found; ++ - 6 to 10 found; +++ - > 10 TLS found in 10 consecutive low-power fields.

To confirm the consistency of grading, the cases were scored independently by two investigators. Examples of TAL and immune cell staining are shown in Figure 1.

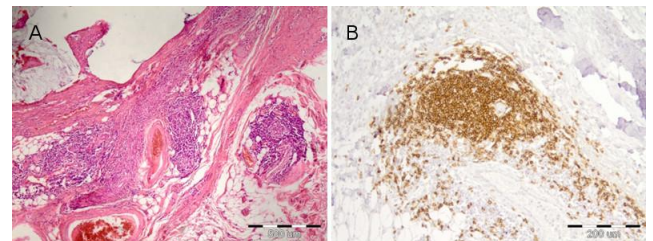


Figure 1: TLS in the invasive front of CRC; A) Well-formed TLS in the invasive front of CRC at the right side of the microscope (10 x 4); B) The same LS immunostained with an antibody against CD20. The cells around the venule are CD20+ (10 x 10)

Descriptive statistical methods were used for statistical analysis of the data. Categorical variables are presented with absolute and relative numbers (%).

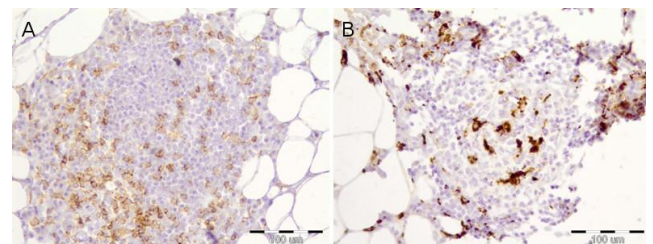


Figure 2: TLS in the invasive front of CRC; A) CD4+ T lymphocytes in the periphery of the follicle (10 x 20); B) CD21+ dendritic cells in the centre of B zone (10 x 20)

Fisher's exact test was used for comparison of categorical variables. Spearman's correlation coefficient was used to determine the degree of correlation between analysed parameters. The statistical program SPSS for Windows, version 19.0 was used.

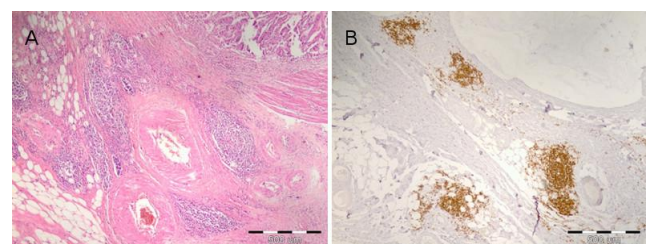


Figure 3: TLS in the invasive front of CRC; A) Five TLS in one low-power field (H.E. 10 x 40); b) CD20+ areas of TLS (CD20 10 x 4)

## Results

The presence of TLS was found in 85 (82.52%) cases of the total of 103, whereas in 18 (17.47%) cases TLS was not found, that is the inflammatory infiltrate which contained a different number of CD20+ lymphocytes confirmed with immunostainings showed no TLS organisation.

Table 1 shows the distribution of TLS in the tumour of the examined patients.

**Table 1: TLS distribution in invasive tumour front in analysed series of 103 patients**

TLS	No of patients	Per cent (%)
0	18	17.47
+ (1-5)	43	41.74
++ (6-10)	27	26.21
+++ (> 10)	15	14.56

For the value of  $p < 0.0001$ , the statistical analysis showed a significant influence of a tumour local growth on the quantity of TLS in the infiltrative front of the neoplasm. Their quantity was significantly larger in tumours of lower T status.

**Table 2: TLS distribution about the examined parameters**

Parameter	Tertiary Lymphoid Structures - TLS					P value
	0		+(1-5)	++(6-10)	+++(>10)	
T status	N	N = 18	N = 43	N = 27	N = 15	
T1	5	0	0	0	5 (100%)	<0.0001
T2	7	0	1 (14.29%)	1 (14.29%)	5 (71.43%)	p (Kruskal-Wallis test)
T3	60	7 (11.67%)	25 (41.67%)	23 (38.33%)	5 (8.33%)	
T4	31	11 (35.48%)	17 (54.84%)	3 (9.68%)	0	
Lymph node involvement						P =
No	56	4 (7.14%)	17 (30.36%)	22 (39.29%)	13 (23.21%)	0.000011**
Yes	47	14 (29.79%)	26 (55.32%)	5 (10.64%)	2 (4.26%)	p (Chi-square test)
Lymphatic invasion – L1						P =
No	54	5 (9.26%)	13 (24.07%)	22 (40.74%)	14 (25.93%)	0.000001**
Yes	49	13 (26.53)	30 (61.22%)	5 (10.2%)	1 (2.04%)	p (Chi-square test)
Stage						< 0.0001
I	11	0	0	1 (9.09%)	10 (90.91%)	
II	42	2 (4.765)	16 (38.1%)	21 (50%)	3 (7.14%)	p (Kruskal-Wallis test)
III	44	12 (27.27%)	26 (59.09%)	4 (9.09%)	2 (4.55%)	
IV	6	4 (66.67%)	1 (16.67%)	1 (16.67%)	0	
Grade of differentiation - G						0.028*
G1	5	0	1 (20%)	1 (20%)	3 (60%)	
G2	83	15 (18.07%)	33 (39.76%)	23 (27.71%)	12 (14.46%)	p (Kruskal-Wallis test)
G3	15	3 (20%)	9 (60%)	3 (20%)	0	

\* $p < 0.05$ ; \*\* $p < 0.01$ .

TLS quantity was significantly different among patients with or without disease spread in the regional lymph nodes ( $p = 0.000011$ ). In patients with involved regional lymph nodes more often than in patients without lymph node metastasis, no tertiary follicles were found (29.79% vs 7.14%). They were also found to have 1 to 5 tertiary follicles (55.32% vs 30.36%).

Patients without or with lymphatic invasion also showed a significant difference regarding the finding of tertiary lymphatic structures ( $p = 0.000001$ ).

Patients with lymphatic invasion more often than those without invasion had no tertiary follicles in the infiltrative front of the neoplasm (26.53% vs 9.26%). They were also more frequently found to have a small number of tertiary follicles, from 1 to 5 when

compared to patients without lymphatic invasion (61.22% vs 24.07%).

The finding of TLS in the infiltrative front of the neoplasm was significantly dependent on the spread of the disease ( $p < 0.0001$ ). A smaller quantity of TLS was found in patients with colorectal carcinoma in the more advanced stage.

Patients with good, moderate and poor tumour differentiation had a significantly different quantity of tertiary lymphoid structures ( $p = 0.028$ ).

The largest quantity of tertiary follicles was found in the group of well-differentiated tumours, that is, in 3/5 (60%) of patients, more than 10 tertiary follicles were found. In the group of moderate and poorly differentiated tumours, the largest number/percentage had from 1 to 5 tertiary follicles - 33/83 (39.76%) and 9/15 (60%) of patients, respectively.

The correlations between TLS and stage of the disease and tumour grade showed that the number of tertiary lymphoid structures had a negative, indirect correlation with the stage of the disease ( $R = -0.635$ ), and with the degree of differentiation ( $R = -0.243$ ). Therefore, the number of tertiary follicles was larger in tumours with lower stage and better differentiation, and vice versa. The two correlations were confirmed to be statistically significant: the association of the number of tertiary follicles with the staging was significant for the value of  $p < 0.0001$ , and with the degree of differentiation for the value of  $p < 0.05$ .

## Discussion

Colorectal cancer (CRC) being the third most common malignant disease in the humans and the second most common cause for the lethal outcome of malignancies in the West European countries and eighth in the developing countries is a major public health problem [6] [9] [10] [11] [12]. Prognosis of colorectal cancer mostly depends on the stage of disease, and the TNM staging (AJCC/UICC) remains the most reliable prognostic indicator for patients with CRC [6]. It also depends on many other factors such as a tumour and surgery-related factors, histological, genetic, loss of heterozygosity at 18q, microsatellite instability status and molecular, protein biomarkers and others factors [6] [13] [14] [15] [16].

During the phases of CRC progression, a different amount of inflammatory cells infiltrate a tumour, among them T and B lymphocytes, macrophages and mast cells [3] [6] [8]. It is considered that inflammatory infiltrate promotes tumour growth, but the immune cells may control cancer progression and outcome. The control of

immune system over tumors is based on the theory of 3E rules i.e. 3-phase interaction between the tumor and host immune system: elimination (immune system eliminates tumour cells), equilibrium (immune system controls a tumour) and escape (tumour cells develop resistance to the immune system) [3] [17].

There are evidences that high amount of tumour-infiltrating cells are more common in CRC with lower stage, in tumours with lower T status-local tumour growth, without nodal involvement and metastases and that CRC progression is influenced by inter-reaction between cancer cells and tumour microenvironment belonging to the patient [3] [18] [19] [20]. Human B cells develop in the bone marrow and after activation by Ag, enter primary follicles of lymph nodes or other lymphoid tissues where they undergo extensive proliferation and differentiation in plasma cells producing antibodies. After tumour antigens are recognised, it is discovered that the majority of patients with cancer develop tumour-specific antibodies [21] [22]. B cells present in a tumour infiltrating lymphocytes (TIL) might directly kill tumour cells through Ab-independent mechanisms or could mediate TIL effects by regulating other immune cells. They can promote the differentiation of Th1 and Th2 cells, facilitate the formation of CD4+ T cells memory, and promote the survival and proliferation of activated CD8<sup>+</sup> T cells [23].

TLS are described to occur in tumour stroma, and invasive tumour front in different types of cancer and a correlation was found between high densities of TLS and prolonged patient's survival in breast cancer, high grade serous ovarian cancer, non-small cell lung cancer, and CRC [5] [23] [24]. Change in the immune response to host aimed at strengthening or rejection of tumour cells can be a solid ground for cancer immunotherapy and can offer an optimal anti-tumour response in patients with malignant tumours including CRC [25]. Also, published data confirm that chemotherapy might stimulate the immune system against a tumour by increasing the density of intra-tumour lymphocytes, which is associated with a reduction of neoplasm and prolonged patient's survival [26].

This study analysed the quantity of TLS regarding elements of the TNM system (T, N) as well as regarding lymph vessels invasion and tumour differentiation. We found a statistically significant difference in the density of TLS in patients with different stage, with or without lymphatic invasion and with different grade. The density of TLS was higher in patients with lower stage, lower T status, without lymph node metastasis and in cancer patients with better differentiation. This finding in our series of analysed patients has shown that the density of TLS plays an important role in the control of tumour growth in the phase of elimination and equilibrium and that it can be a parameter for the progression of a neoplasm in CRC patients. The density of TLS influences the control of tumour progression.

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