https://doi.org/10.48047/AFJBS.6.11.2023.1785-1793

AfricanJournalofBiological Sciences

Research Paper Open Access

Relationship of peritumoral Tumor Infiltrating Lymphocytes with Tumor Budding in Colorectal Adenocarcinoma

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Article Info

Volume 6, Issue 11, May 2024

Received:10 Jun 2024

Accepted :20 Jul 2024

Published: 23 Aug 2024

doi:10.48047/AFJBS.6.11.2023.1785-1793

ABSTRACT: Colorectal cancer is the third most common cancer in men and the second most common cancer in women worldwide. Peritumoral lymphocytic reaction is associated with prognosis in tumor budding. The aim of this study was to analyze Tumor Infiltrating Lymphocytes (TILs) in colorectal adenocarcinoma and correlate it with tumor budding in colorectal adenocarcinoma. This study used a cross-sectional design. Seventy slides of colorectal adenocarcinoma were stained with hematoxylin and eosin (H&E). The relationship between TILs and tumor budding of colorectal adenocarcinoma was statistically analyzed using the Chi Square test. From 70 samples examined, TILs were assessed and categorized into low grade, intermediate grade and high grade then correlated with tumor budding. Tumor budding was assessed and then categorized into low grade and high grade. In low-grade budding tumor samples with high TILs of 5 (83.3%), intermediate TILs of 22 (71.0%) and low TILs of 11 (33.3%). In contrast, in high-grade budding tumor samples with high TILs of 1 (16.7%), intermediate TILs of 9 (29.0%) and low TILs of 22 (66.7%). Thus, the TILs score was significantly higher in low-grade budding tumor samples (p=0.003). TILs can be used as a good prognostic factor in colorectal adenocarcinoma.

Keywords: Colorectal Adenocarcinoma, Tumor Infiltrating Lymphocytes, Tumor Budding

Introduction

Colorectal cancer is the third most common cancer in the world. The incidence is higher in men than women and is rare in people under the age of 40. Although surgical and chemotherapy approaches for colorectal cancer have advanced, the prognosis is still poor because one-third of patients who undergo curative resection die within five years of surgery (Effendi & Rey, 2018; Testa, 2018; Testa, 2018). The

World Health Organization (WHO) in 2018 explained that there were 1.80 deaths. million new cases of colorectal cancer were reported globally and 862,000 patients died from colorectal cancer (Ahmed, 2020). More than 1.9 million new cases of colorectal cancer and 904,000 deaths are expected to occur in 2022, representing almost one in 10 cancer cases and deaths. Colorectal cancer ranks third in terms of incidence but second in terms of mortality (Bray et al., 2022). GLOBOCAN data in 2020, the incidence of new colorectal cancer in Indonesia was 33,427 cases or around 8.4% of the total 396,914 cancer cases (American Cancer Society (ACS), 2020). Many factors affect the prognosis of colorectal carcinoma patients such as tumor growth characteristics, including the presence of vascular or lymphatic invasion (both mural and extramural) and the size and grade of the tumor. Metastasis to the peritoneum, lymph nodes, and liver is the main cause of death in colorectal cancer (Noffsinger, 2017; Wang et al., 2018).

A unique characteristic of malignant neoplastic cells is their ability to invade new tissues and metastasize. This process begins with the dissociation of some cells from the front of the tumor invasive area. This is associated with transformation, dedifferentiation or transdifferentiation of cells known as the phenomenon of tumor budding. Tumor budding is a single cell or a group of up to four cells at the front of tumor invasion, indicating epithelial-mesenchymal transition (EMT) which is identified as a very poor prognosis subgroup (Lino-Silva, 2018). The presence of increased tumor infiltrating lymphocytes (TILs) is established as a positive prognostic factor in malignancies including colorectal carcinoma (Miskad et al., 2020). The immune response in colorectal cancer associated with the presence of effector memory T cells has been shown to correlate with cancer histopathology and has revealed significant prognostic value (Zadka, 2021).

Patients with a percentage of TILs >5% have a significantly longer survival compared to the low TILs group (Brummel, 2023). Immunohistochemical detection of CD103+ TILs can provide a goal to assess intraepithelial TILs. Showing a strong correlation between CD103+ TILs and survival in high-grade serous ovarian carcinoma without the need to distinguish epithelial and stromal locations (Webb et al., 2014). In 2014, the International TILs Working Group (ITWG) recommended standardization of the approach to measure TIL density in breast cancer. They showed that there is a way to evaluate TIL density in HE-stained sections without evaluating lymphocyte groups. In order to provide individual therapy according to risk stratification, biomarkers that can predict prognosis must be found to improve prognosis. In addition to tumor factors, the tumor environment (i.e. extracellular matrix, immune cells, and cytokines) plays an important role in tumor growth, invasion, metastasis, and proliferation (Iseki et al., 2018).

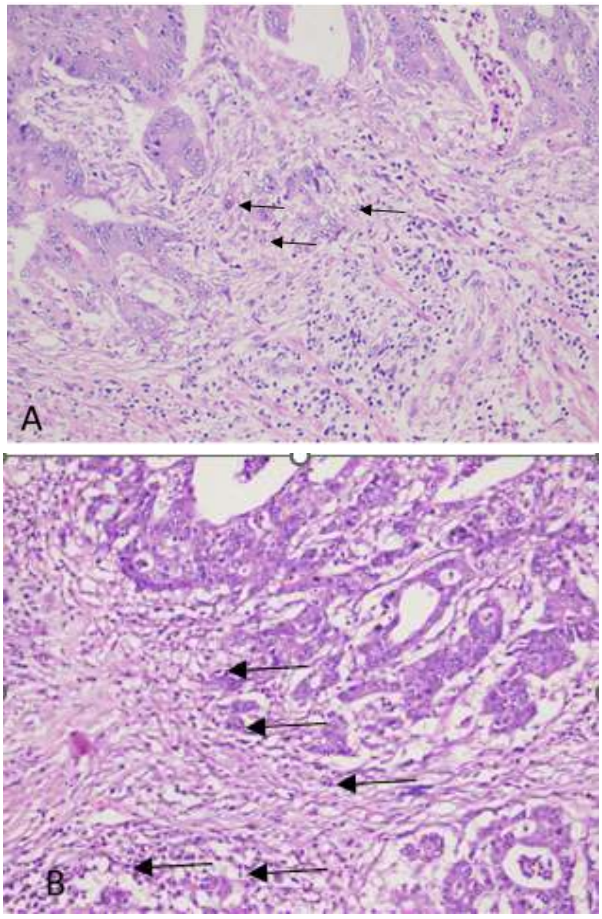
Tumor budding morphology shows cellular changes identified as intact cells or small cytoplasmic fragments originating from solid or glandular structures at the front of tumor invasion and numbering one to four cells. These changes are well organized with the basal lamina bordering the outer edge of the tumor invasion. Tumor budding is the movement of single cells originating from the main tumor cluster growing into the stroma. The budding tumor has a sheath of microvilli and myofibroblasts indicating that tumor invasion occurs through dendritic extensions, called podia or tubular invasion poles that are part of the tumor. However, this situation can be focally disturbed, where myofibroblasts can be lost or absent. Structurally, there is no basal lamina, and the cytoplasm of tumor cells is in direct contact with the extracellular matrix (Cho, 2018). TILs trigger TCR and TGF-β receptors at the tumor site (Qiu, 2021).

Several studies have shown peritumoral lymphocytic infiltration to be an independent prognostic factor in colorectal carcinoma. It was reported that the presence of intratumoral budding was strongly correlated with peritumoral budding, but was found to be an independent prognostic factor in multivariate analysis. Peritumoral lymphocytic reaction was associated with prognosis in tumor budding, indicating that the immune response targets tumor budding (Miskad et al., 2020). Therefore, this study assessed whether TILs correlate with tumor budding in colorectal adenocarcinoma, so that it can be one of the candidate prognostic biomarkers for colorectal adenocarcinoma.

Methods

From January 2020 to December 2023, we obtained 70 slide samples from patients diagnosed with colorectal adenocarcinoma at the Dr. Wahidin Sudirohusodo Anatomical Pathology Laboratory, Faculty of Medicine, Hasanuddin University, and the Makassar Pathology Diagnostic Center. Tumor budding

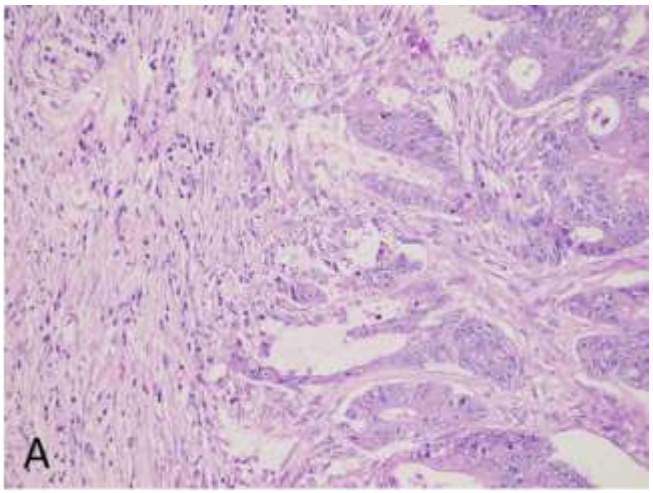
evaluation was performed on hematoxylin and eosin (H&E) slides of the entire area. Tumor budding was analyzed as a continuous variable and categorized into two groups: low grade (1-9 buds/0.785 mm2) and high grade (10 buds/0.785 mm2).

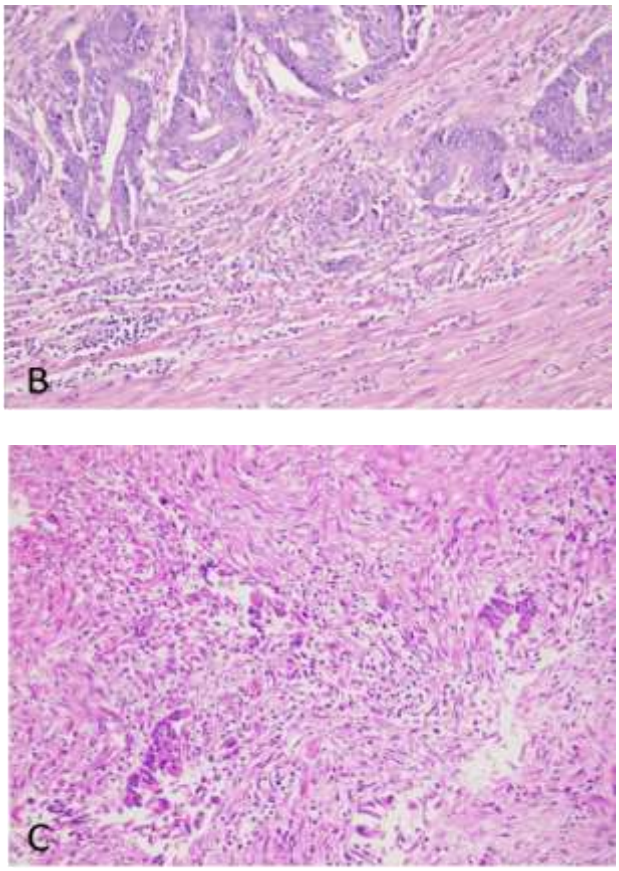


A, low grade; B, High grade (200x Magnification).

Figure 1. Tumor Budding in Colorectal Adenocarcinoma

Stromal TILs evaluation was performed on hematoxylin and eosin (H&E) slides of the entire peritumoral area following the 2014 International TILs Working Group guidelines.(13) TILs were analyzed as a continuous variable and categorized into three groups: high TILs (> 55%), intermediate TILs (15-50%), and low TILs (≤10%).





A, Low ; B, Intermediate; C, High; (200x Magnification)

Figure 2. TILs score in colorectal adenocarcinoma

Using a 400x light microscope. The assessment was performed by two pathologists who were blinded to the data and clinical outcomes.

The Statistical Program for Social Sciences (SPSS) 27 for Windows was used to process the data for this investigation. To evaluate the correlation between categorical variables, the chi-square test was used.

Results and Discussion

Table I. Sample Characteristics

Characteristics		n	%
Sex	Male	37	52,9
	Female	33	47,1
Age	< 50 Years	21	30,0
	≥ 50 Years	49	70,0
Tumor Budding	Low	38	54,3
	High	32	45,7
TILs	Low	33	47,1
	Intermediate	31	44,3
	High	6	8,6

Table I shows the distribution of 70 colorectal adenocarcinoma samples based on age, sex, tumor budding and tumor infiltrating lymphocyte (TILs) grade scores. This study involved a total of 70 samples. Among the samples, 21 (30%) were less than 50 years old, while 49 (70%) were 50 years old or older. There were 37 male patients (52.9%) and 33 female patients (47.1%). There were 38 samples with low-grade tumor budding (54.3%), and 32 samples with high-grade tumor budding (45.7%). There were 33 samples with low-grade TILs (47.1%), 31 samples with intermediate-grade TILs (44.3%) and 6 samples with high-grade TILs (8.6%).

Table 2. shows that of the 70 samples of colorectal adenocarcinoma

TILS		Tumor Budding		Total	p Value
		Low	High		
Low	n	11	22	33	0,003
	%	33,3%	66,7%	100,0%	
Intermediate	n	22	9	31	
	%	71,0%	29,0%	100,0%	
High	n	5	1	6	
	%	83,3%	16,7%	100,0%	
Total	n	38	32	70	
	%	54,3%	45,7%	100,0%	

Table 2 shows that from 70 samples of colorectal adenocarcinoma. In low-grade tumor budding samples with high TILs of 5 (83.3%), low-grade tumor budding samples with intermediate TILs of 22 (71.0%) and low-grade tumor budding samples with low TILs of 11 (33.3%). In contrast, in high-grade tumor budding samples with high TILs of 1 (16.7%), high-grade tumor budding samples with intermediate TILs of 9 (29.0%) and high-grade tumor budding samples with low TILs of 22 (66.7%). Based on these data, the TILs value was significantly higher in low-grade tumor budding samples ($p = 0.003$).

The presence of increased tumor infiltrating lymphocytes (TILs) as a marker of immune activation by tumors, has consistently been shown to be an independent predictor of better prognosis in various malignancies, including colorectal carcinoma. The presence of cytotoxic CD8+ T cells in tumors has a strong association with positive outcomes in various malignancies. Epithelial tumors may be infiltrated by Resident Memory (TRM) T cells with transmembrane heterodimer complexes that mediate cell adhesion, migration, and homing of lymphocytes through interactions with E-cadherin (Kim, 2019). T lymphocytes in the epithelium, especially CD8+ T cells (including cytotoxic cells), T cells (CTLs) and TRM cells) and CD4+ regulatory T cells (Mami-Chouaib et al., 2018). E-cadherin can be downregulated in malignant tumors undergoing epithelial-mesenchymal transition (EMT), a process observed in most cancers, including those with low immunogenicity such as cholangiocarcinoma, ovarian cancer, and breast cancer (Van den Bulk, 2019).

The immune system can recognize and destroy tumor cells through T cell-mediated mechanisms. Therefore, various therapeutic approaches focus on enhancing and/or restoring T cell function in cancer patients. An effective immune response involves the concerted action of several different cell types, among which CD8 T cells are key players that can specifically recognize and kill cancer cells through the release of cytotoxic molecules and cytokines. A percentage of tumor-infiltrating CD8 T cells (CD8 TILs) recognize tumor-associated antigens, which include overexpressed antigens, as well as tumor-specific neoantigens, which arise as a result of tumor-specific mutations. Tumor-specific CD8 T cells are located in the lymph nodes and then migrate via the blood to the tumor, where they perform their effector functions. CD8 TILs represent a heterogeneous cell population consisting of tumor-specific T cells. T cells are recruited to tumor sites through inflammation associated with tumor development. Recruitment and retention within tumors require T cells to express a specific set of chemokine receptors and integrins. Among integrins, integrin αE, also known as CD103, is expressed on a subset of dendritic cells in the gut and a population of T cells found in peripheral tissues, known as tissue-resident memory (TRM) T cells. CD8 TILs in several solid tumors are known to be upregulated by TGF-β. These cells have a TRM

phenotype, have a high frequency of tumor-reactive cells, have a distinct TCR repertoire, and are able to recognize and kill autologous tumor cells (Duhen et al., 2018).

TRM cells also express high levels of granzyme B, IFN, and TNF that support their cytotoxic features. Within tumor cells, natural TRM cells directly control tumor growth. Infiltration of various cancer types by TRM cells correlates with better clinical outcomes (Hoffmann & Smyth, 2021). TILs are strongly associated with improved overall survival (OS), disease-free survival (DFS), and/or relapse-free survival (RFS) in most cancer types, including urothelial cell carcinoma, ovarian cancer, cervical cancer, endometrial cancer, breast cancer, colorectal cancer, gastric cancer, and head and neck cancer (Brummel, 2023).

One of the histomorphological features of EMT is tumor budding, which serves as an additional prognostic factor in colorectal cancer. Tumor budding is defined as a single tumor cell or a small group (up to five cells) at the invasive tumor front. Tumor characteristics associated with aggressive behavior include lymph node metastasis, poor differentiation, vascular/lymphatic invasion, and recurrence. Tumor budding is another important prognostic factor. Epithelial cells undergoing EMT acquire the ability to proliferate and form tumor buds within the tumor stroma. Following the establishment of tumor budding and metastatic potential, interactions with lymphocytes in the tumor stroma become essential for the immune response to combat tumor invasion.(8) An important molecular feature of EMT is the downregulation of E-cadherin, a cell adhesion molecule present in the cell membrane of most normal epithelial cells. E-cadherin acts as a tumor suppressor by inhibiting invasion and metastasis, and is frequently suppressed or degraded during transformation. Although a number of different molecular processes are involved in initiating EMT, reduced E-cadherin expression is thought to be the final molecular step in the transformation of epithelial cancer cells into mesenchymal cells, identifying a role for E-cadherin in tumor budding and also the association of E-cadherin expression with EMT-related proteins that may play an important role in tumor nest development (Yamada et al., 2016).

High-grade colorectal adenocarcinoma, characterized by poor prognosis, is often associated with tumor budding. This mesenchymal-like subtype is characterized by overexpression of stem cell markers, neoangiogenesis, and activation of the TGF-β and WNT/β-catenin signaling pathways that modulate immune cell evasion and metastasis. Activated Beta catenin signaling pathways can interfere with anti-tumor immunity by interfering with dendritic cell activation and causing T cell death. High-grade tumor budding has an inverse correlation with the presence of immune cell infiltration in the front area of tumor invasion (Wei, 2020).

In our study, statistical data showed a significant relationship between TILs and tumor budding (Table II). The sample distribution showed that the TILs score was higher in low-grade tumor budding compared to high-grade. This correlates with Lei Wei's study which found that high-grade tumor growth has an inverse correlation with immune cell infiltration at the invasive tumor front (Wei, 2020). In addition, overexpression of stem cell-related genes and activation of WNT and TGFβ signaling have been shown to be expressed in growing tumors with CTNNB1 mutations that cause impaired tumor immunity. Activated β-catenin signaling pathways can interfere with antitumoral immunity by disrupting dendritic cell recruitment to the TME and causing T cell death. This is most likely due to immune cells that have many similarities with tumor cell migration and are regulated by chemokines and their receptors (Kang et al., 2021). In addition, T lymphocyte migration in tumor tissue is known to be influenced by chemokine receptors (Franciszkiewicz et al., 2009). Integrins expressed on T cells residing in the tumor microenvironment (TME), where they are found on mucosal CD8+ T lymphocytes, especially intraepithelial lymphocytes (IELs). Their expression is induced in tumor-specific CD8+ T cells through simultaneous signals from the TGF-β receptor (TGFBR) and the T cell receptor (TCR), triggered by TGF-β and MHC class I (MHC-I)/tumor peptide complex (Qiu, 2021). These findings also support the study by Lugli and Lang-Schwarz who found that the ratio of lymphocytes to tumor budding is a good prognostic factor in patients with colorectal tumors (Qiu, 2021; Lugli et al., 2012). This study also reported that TILs and tumor budding can predict long-term prognosis in colorectal cancer. We found that the density of low TILs was higher in high-grade tumors compared to low-grade tumors. This finding correlates with Lei Wei's study showing that high-grade tumor growth has an inverse correlation with immune cell infiltration at the invasive tumor front (Wei, 2020). In addition, overexpression of stem cell-related genes

and activation of WNT and TGFβ signaling have been shown to be expressed in growing tumors. Activated β-catenin signaling pathways can interfere with antitumoral immunity by disrupting dendritic cell recruitment to the TME and causing T cell death. Supporting this, a study conducted by Zhijuan Qiu showed that tumor cells also upregulate the expression of FABP4 and FABP5, thereby defeating tumor-reactive CD8 TRM cells and causing TRM cell death, so it can be said that cancer development can suppress T cells. immunity (Qiu, 2021). However, it is important to consider that tumorigenicity and the area of tumor microinvasion can vary based on genetic background and etiology. This variation can lead to differences in the characteristics of each type of tumor. As highlighted by Kim, the correlation between immune cells and prognosis can vary in different tumors (Kim, 2019).

Kang et al's study found significant TILs in young age, tumor size, and low-grade histopathology (Kang et al., 2021). The survival rate of patients with low grade was better than those with high grade. Tumors with low and high differentiation grades had significantly larger peritumoral lymphocytes than poorly differentiated tumors. A hallmark of cancer is the evasion of immune cells that allows tumor cells to avoid destruction by the immune system (Loi et al., 2019). Christine's study showed that intratumoral and peritumoral lymphocyte densities varied in each case and no relationship was found between immune cell infiltration and tumor grade (Sanders et al., 2022).

Conclusion

In conclusion, our study found a significant association between TILs and tumor budding in colorectal adenocarcinoma. Patients with low-grade tumor budding showed higher TILs density compared with patients with high-grade tumor budding. These findings suggest that TILs may serve as a prognostic factor in colorectal adenocarcinoma. In addition, TILs may influence the development of tumor budding in colorectal cancer.

Conflict of Interest

The authors declare that they have no conflicts of interest with any financial organizations regarding the materials discussed in the manuscript.

Funding

The authors report no sponsorship involvement in the study that could have influenced the results of this study.

Authors' Contributions

Emi Sumarya and Upik Anderiani Miskad contributed equally to this study, Emi Sumarya, Upik Anderiani Miskad and Muhammad Husni Cangara contributed greatly to the research design and data investigation, Emi Sumarya, Upik Anderiani Miskad and Mardiati in data collection, Syarifuddin Wahid, Djumadi Achmad and Suryani Tawali for data analysis, Emi Sumarya, Upik Anderiani Miskad and Muhammad Husni Cangara for drafting the manuscript. All authors read and approved the final version of the manuscript.

Acknowledgements

The authors would like to thank Upik Anderiani Miskad and Muhammad Husni Cangara for their correction activities.

Participating Researchers

The authors would like to thank Syarifuddin Wahid and Djumadi Achmad who served as scientific supervisors.

Participating Researchers

The authors would like to thank Upik Anderiani Miskad and Mardiati who collected the data.

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