Microsatellite Status, Tumor Budding, CD3 and CD8 T Cell Densities in Relation to Invasiveness, Lymph Node Involvement in Colorectal Adenocarcinoma

Rebecca Noerjani Angka[1,2*], Aru Wisaksono Sudoyo[3], Nurjati C. Siregar[4*], Wifanto Saditya Jeo[5], Melva Louisa[6], Demak Lumban Tobing[7]

[1]Doctoral Program of Biomedical Science, Faculty of Medicine Universitas Indonesia, Jakarta, Indonesia.
[2]Department of Anatomical Pathology, Faculty of Medicine and Health Sciences, Krida Wacana Christian University (UKRIDA), Jakarta, Indonesia.
[3]Division of Hematology and Medical Oncology, Department of Internal Medicine, Faculty of Medicine Universitas Indonesia - Cipto Mangunkusumo Hospital, Jakarta, Indonesia.
[4]Department of Anatomical Pathology, Faculty of Medicine Universitas Indonesia - Cipto Mangunkusumo Hospital, Jakarta, Indonesia.
[5]Department of Surgery, Digestive Division, Faculty of Medicine, Universitas Indonesia - Cipto Mangunkusumo Hospital, Jakarta, Indonesia.
[6]Department of Pharmacology and Therapeutics, Faculty of Medicine Universitas Indonesia, Jakarta, Indonesia.
[7]Department of Clinical Pathology, Dharmais Cancer Hospital, Jakarta, Indonesia.

Corresponding authors:
Prof. Aru Wisaksono Sudoyo, MD., PhD. Division of Hematology and Medical Oncology, Department of Internal Medicine, Faculty of Medicine Universitas Indonesia - Cipto Mangunkusumo Hospital. Jl. Diponegoro no. 71, Jakarta 10430, Indonesia. Email: arusudoyo@gmail.com
Rebecca Noerjani Angka, MD., PhD. Department of Anatomical Pathology, Faculty of Medicine and Health Sciences, Krida Wacana Christian University (UKRIDA). Jl. Arjuna Utara no. 65, Kebon Jeruk, Jakarta 11510, Indonesia. Email: rebecca.norjani@ukrida.ac.id.

ABSTRACT

Background: *Aside from the factors more commonly known as predictors in colorectal cancer, there are 3 additional less well-known factors, i.e., tumor budding (TB), T cell densities and loss of MMR protein expression, the aforementioned three factors are known to be independent predictive factors in CRC survival. In this study association of TB, T cell densities and loss of MMR protein were examined to see the association with differentiation, tumor location, invasiveness and lymph node invasiveness.* ***Methods:*** *A retrospective cohort study was conducted using 68 CRC Formalin Fixed Paraffin Embedded samples from patients who underwent removal surgeries with the diagnosis of adenocarcinoma not otherwise specified. TB counts were identified by immunohistochemical staining using Pan-Cytokeratin AE1/AE3 and were categorized into low and high. MMR protein loss was analyzed using antibodies MLH1 and MSH6 categorized as positive and negative, then classified into Microsatellite Stable (MSS) and Microsatellite Instability (MSI). CD3 and CD8 T cell densities were identified using CD3 Biocare Medical and CD8 Biocare, was categorized into low and high. Secondary data from medical records were collected and analyzed using SPSS 25.* ***Results:*** *A significant relationship was found between tumor budding with the depth of invasion and lymph node involvement (p=0.021 and 0.020).* ***Conclusion:*** *Tumor budding (TB) plays a role in the depth of invasion and lymph node involvement in CRC but has no significant relationship with CD3/CD8 densities, differentiation, location, and MMR status. There was also no significant relationship between MMR status with differentiation, location, depth of invasion, lymph node involvement, and TB.*

Keywords: *lymph node involvement, MMR, T cell densities, tumor budding, tumor differentiation.*

INTRODUCTION

Colorectal cancer (CRC) is the fourth most frequently diagnosed cancer in Indonesia, with 408,661 new cancer cases and 242,988 deaths.[1] Tumor budding (TB) according to the International Tumor Budding Consensus Conference (ITBCC) as an independent factor for predicting lymph node metastasis in pT1 colorectal cancer, serves as an early manifestation of histological invasion and metastasis along outwardly invasive tumors, and is an independent prognostic factor in colorectal cancer. TB is associated with high tumor-nodes-metastasis (TNM) stage, high tumor grade, presence of lymphovascular invasion, lymph node involvement, and distant metastasis.[2, 3] Patients with the same TNM status do not always give the same outcome. Therefore tumor budding is one option that is starting to be used as an independent predictive factor.[4,5] According to Smedt, the presence of TB is currently used to compare survival and outcome [6]. Another factor that influences cancer development is the patient's immunity. Previous studies have found that there are several types of immune cells, including CD3 and CD8 T cells, which are considered prognostic factors.[7] We examined CD3 and CD8 T cell densities in this study to know the immune status of patients. Deficient MMR (dMMR) results in a strong mutator phenotype known as microsatellite instability (MSI), that recognized as one of the major ones CRC carcinogenetic pathway,[8] so MMR status needs to be known to determine the patient's prognosis.[9,10] Study on invasiveness, differentiation, location and lymph node involvement in relation to TB was conducted by Fujiyoshi and Marx.[2,11]

The aim of this study is to determine whether there is a relation between the depth of tumor invasion, differentiation, location, lymph node involvement with TB, CD3 and CD8 T cell densities, and MMR status.

METHODS

This study is an observational, cross-sectional, retrospective study of all colorectal cancer patients of all stages, who underwent tumor removal surgery between 2015-2019 at MRCCC Siloam Semanggi and RSUPN Dr. Cipto Mangunkusumo, both in Jakarta. This study was approved by the Ethics Committee of the University of Indonesia no. KET-349/UN2. F1/ETIK/PPM.00.02/2022 dated 11 April 2022.

Patients and Tissues

All clinical data of CRC patients from anatomical pathology archives were retrieved. Histology picture and report were reassessed by NCS (member of the team) and only adenocarcinoma NOS were selected. All 68 Formalin Fixed Paraffin Embedded (FFPE) samples that fulfill the sample criteria: patients >18 years old who have not undergone any treatment other than surgery were selected.

The depth of invasion was categorized into pT1-pT2, pT3-pT4,[12] tumor differentiation was categorized into a well (well-moderate) and poor, and location tumor was categorized into the right (cecum, ascending colon, hepatic flexure, and 2/3 proximal of transverse colon) and left 1/3 distal of transverse colon, splenic flexure, descending colon, sigmoid colon, and rectum), whereas lymph node involvement was categorized into no and yes (whether there are metastases or not to lymph node) following American Joint Committee on Cancer (AJCC) Cancer Staging 8th Edition.[13]

Immunohistochemistry of TB, CD3 and CD8 T Cell Densities, and MMR Protein Expression

Immunohistochemical staining to visualize TB was done on a 3 µm specimen, using Pan-Cytokeratin AE1/AE3 (CM011B) from Biocare Medical® in 1:100 dilution, following manufacturer instruction. TB is counted in the peritumoral in the invasive part using a microscope Olympus CX23 (field diameter 20 mm, field area 0.785 mm^2). We scanned 10 individual fields with low magnification (100x) to identify the hotspot and only one selected hotspot was counted using 200x times magnification. The TB result is divided by the normalization factor of 1.00^3. TB is categorized into 2 values: low (Bd, 0-4 buds/0.785 mm^2) and high (Bd $\geq$ 5 buds/0.785 mm^2).[14] TB is calculated by 2 anatomical pathologists (NCS and ID) and researchers (RNA). IHC to visualize CD3 and CD8 T cells was done by using CD3

Biocare Medical (catalog ACR324B) in 1:50 dilution, and CD8 using CD8 Biocare Medical (catalog 108R-14) in 1:250 dilution, following manufacturer instruction. Tonsil was used as a positive control for the staining of tumor budding and T cells. CD3 and CD8 T cell densities were categorized into low and high according to the cut-off point.[15] The samples were scanned using the Leica Aperio AT2 Slide Scanner. IM was determined 360µm from the tumor edge into the tumor and 360µm into healthy tissue, after which the densest area containing CD3 cells was selected in an area of 1 mm2. CD3 cells in CT and IM areas were counted using QuPath software.[16] The same steps are performed on the CD8 specimen. IHC staining to visualize MLH1 and MSH6 using MLH-1 (CM220BK) Biocare Medical® in 1:50 dilution and MSH-6 (CM265AK) Biocare Medical® in 1:100 dilution, following manufacturer instruction. The adenocarcinoma colon that has been tested previously was used as a positive control for the staining. The staining results are categorized as positive and negative, then defined as pMMR if both MLH-1 and MSH-6 are positive and dMMR if one or both MLH-1 and MSH-6 are negative.[17] Image processing for TB and MMR status was done using an Indomicro HDMI camera.

Statistical Analysis

Characteristics of the patients were provided in descriptive data. The relationship between TB and MMR status with differentiation, location, depth of invasion, and lymph node involvement, as the relationship between CD3 and CD8 densities with differentiation, location, depth of invasion, lymph node involvement, TB and MMR status were analyzed using chi-square test or Fisher's test in SPSS 25. The results are considered significant when the p-value is less than 0.05.

RESULTS

Characteristics of Colorectal Adenocarcinoma Patients

In this study, 39 (60%) men and 29 (40%) women were found with 38 (55.9%) cases over 50 years old compared to 30 (44.1%) cases under 50 years. The earliest age when the diagnosis was 24 years in the male group and 28 years in the female group. We found 51 (76.5%) cases with pT3-pT4, a majority of 43 cases with pT3, 42 (61.8%) samples with TB ≥ 5 buds, 63 (92.6%) cases with good differentiation, 60 (88,2%) cases with left-sided location and 37 (54.4%) cases with involvement lymph node (**Table 1**). We found 26 (38.2%) cases with tumor budding 0-4 (**Figure 1A**) and 42 (61.8%) cases with tumor budding ≥5 buds (**Figure 1B**). 7 (10.3%) cases with TB 0, 6 cases with good differentiation, and 1 case with moderate differentiation which we classify into good differentiation, 1 case with pT3, and 1 case with lymph node involvement. We found there were 3 (4.4%) cases pT1 where 2 cases with TB 0 and 1 case with TB ≥5 buds, all three without lymph node involvement, well differentiation, and left-sided location. Twelve cases out of 68 cases (17.6%) were MLH-1 negative, 7 cases out of 68 (10.3%) cases were MSH-6 negative and only 4 cases out of 68

Table 1. Characteristics of CRC Patients (n = 68)

Parameter	n (%)
Sex	
Male	39 (57.4)
Female	29 (42.6)
Age (year)	
18-50	30 (44.1)
>50	38 (55.9)
pT	
pT1-pT2	17 (25.0)
pT3-pT4	51 (75.0)
Tumor Budding	
0-4	26 (38.2)
≥5	42 (61.8)
Tumor differentiation	
Well	63 (92.6)
Poor	5 (7.4)
Location	
Right	8 (11.8)
Left	60 (88.2)
Lymph node metastases	
No	31 (45.6)
Yes	37 (54.4)
Microsatellite status	
MSS	53 (77.9)
MSI	15 (22.1)
CD3 T cell densities	
Low	25 (36.8)
High	43 (63.2)
CD8 T cell densities	
Low	23 (33.8)
High	45 (66.2)

(5.9%) cases were MLH-1 and MSH-6 negative. We found only 15 cases out of 68 (22.1%) cases were MSI. We calculated the cut-off value using receiver operator curve (ROC) and found the cut-off value for CD3 IM was 1029/mm^2, for CD3 CT was 959/mm^2, for CD8 IM 730.5/mm^2, for CD8 CT 596.5/mm^2. 4 categories were found in this study: low CT and IM, high CT and low IM or low CT and high IM which were categorized as intermediate, and high CT and IM. Then intermediate category is included in the high classification. As a result, we found 25 (36.8%) cases of CD3 low, 43 (63.2%) cases of CD3 high, 23 (33.8%) cases of CD8 low, and 45 (66.2%) cases of CD8 high.

Relationship Between TB and MMR Status with Tumor Differentiation, Location, the Depth of Invasion and Lymph Node Involvement

In the chi-square analysis, there is a significant relationship between TB with a depth of invasion (p 0.021, OR 4.400, 95%CI 1.375-14.076), and lymph node involvement (p 0.020, OR 3.778, 95%CI 1.346-10.600). No significant relationship between TB with differentiation (p 0.642, OR 2.632, 95%CI 0.278-24.935), location (p 0.700, OR 1.622, 95%CI 0.291-9.042), and MMR status (p 0.288, OR 2.222, 95%CI 0.695-7.109). We found no significant relationship between MMR status with differentiation (p 0.067, OR 0.157, 95%CI 0.024-1.045), location

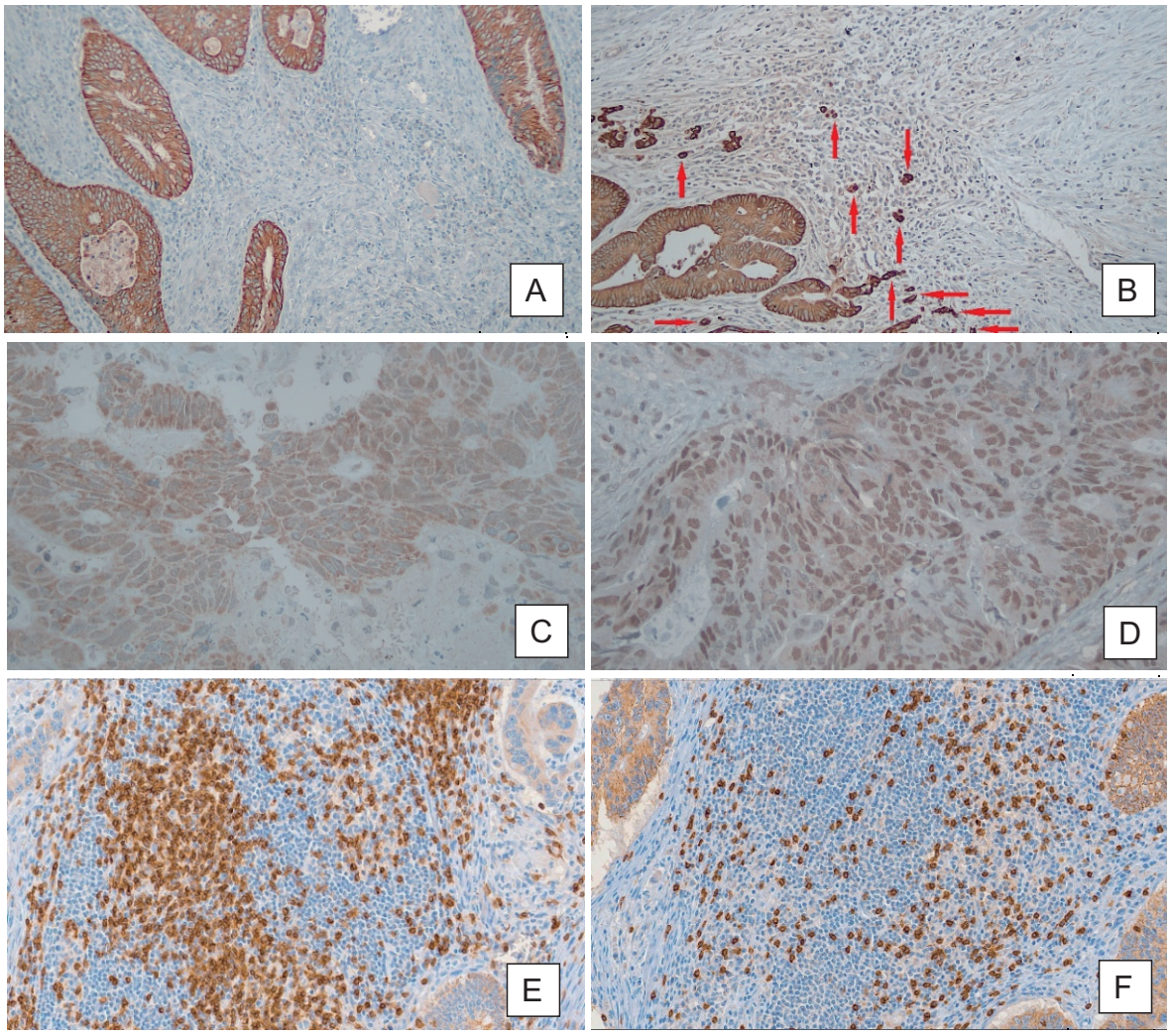

Figure 1. A. Tumor budding in colorectal adenocarcinoma was identified by immunohistochemical staining using Pan-Cytokeratin AE1/AE3 with 200x magnification (brown). There is no tumor budding in this picture. B. There are 4 buds tumor budding. C. Microsatellite status in colorectal adenocarcinoma was identified by immunohistochemical staining. MLH-1 using MLH-1 (concentrated and prediluted monoclonal antibody). D. MSH-6 using MSH-6 (concentrated and prediluted monoclonal antibody). Both with 400x magnification. E. CD3 densities were identified by immunohistochemical staining using CD3 Biocare Medical. F. CD8 using CD8 Biocare Medical.

(p 0.645, OR 0.677, 95%CI 0.118-3.899), depth of invasion (p 0.092, OR 0.165, 95%CI 0.020-1.365) and lymph node involvement (p 0.329, OR 2.114, 95%CI 0.657-6.801). Data is shown in **Table 2**.

Relationship Between CD3 and CD8 Densities with Differentiation, Location Tumor, the Depth of Invasion, Lymph Node Involvement, and Microsatellite Status

In our study, we found that there are no relationship between CD3 and CD8 densities with differentiation (p=0.349, OR 2.795, 95%CI 0.434-18.005 and p=1, OR 1.333, 95%CI 0.207-8.602), location tumor (p=0.409, OR 2.540, 95% CI 0.519-12.422 and p=0.681, OR 1.538, 95%CI 0.314-7.536), the deep of invasion (p=0.885, OR 0.779, 95%CI 0.253-2.397 and p=0.882, OR 1.309, 95%CI 0.398-4.307), lymph node involvement (p=0.959, OR 0.858, 95%CI 0.319-2.305 and p=1, OR 1.138, 95%CI 0.414-3.127), TB (p=1, OR 0.889, 95%CI 0.323-2.444 and p=0.495, OR 1.670, 95%CI 0.575-4.856) and MMR status (p=0.538, OR 1.805, 95%CI 0.507-6.425 and p=0.331, OR=2.424, 95%CI 0.609-9.652). Data is shown in **Table 3**.

DISCUSSION

We found no significant relationship between MMR status with tumor differentiation (p 0.067, 95%CI 0.023-1.045), the depth of invasion (p=0.092, 95%CI 0.020-1.365), location (p=0.645, 95% CI 0.118-3.899) and lymph node involvement (p=0.329, 95% CI 0.657-6.801). Contrary to Jang's study which found a significant relationship between MMR status with tumor differentiation (p=0.011), poor differentiation were more common in MSI-H, also with the depth of invasion (p=0.047), location (p <0.001) and lymph node involvement (p=0.040).[18] Karlberg found that dMMR CRC was more located in the proximal colon.[19] Similarly to Andersen HS, who found that on the right side are more MSI tumors and that the number of lymph nodes involvement was higher in MSI tumors and MSI were associated with a lower risk of TB.[20] By categorizing the tumor location according to their parts, Topal proved that there was a significant relationship between location and the presence or absence of MSI, also with differentiation (p=0.001). Similarly, with our study, Topal found no significant relationship

Table 2. Relationship between TB and MMR status with differentiation, location, depth of invasion, and lymph node involvement

	TB ≥5	TB 0-4	Total (%)	p (95%CI)	pMMR	dMMR	Total (%)	p (95%CI)
Differentiation								
Poor	4	1	5 (7.4)	0.642	2	3	5 (7.4)	0.067
Well	38	25	63 (92.6)	(0.278-24.935)	51	12	63 (92.6)	(0.024-1.045)
Location								
Right	5	2	7 (10.3)	0.700	5	2	7 (10.3)	0.645
Left	37	24	61 (89.7)	(0.291-9.042)	48	13	61 (89.7)	(0.118-3.899)
Depth of invasion								
pT3-pT4	36	15	51 (75.0)	0.021	37	14	51 (75.0)	0.092
pT1-pT2	6	11	17 (25.0)	(1.375-14.076)	16	1	17 (25.0)	(0.020-1.365)
Lymph node								
Yes	28	9	37 (54.4)	0.020	31	6	37 (54.4)	0.329
No	14	17	31 (45.6)	(1.346-10.600)	22	9	31 (45.6)	(0.657-6.801)
Microsatellite status								
pMMR	35	18	53 (77.9)	0.288				
dMMR	7	8	15 (22.1)	(0.695-7.109)				

TB: tumor budding, MSS: microsatellite stable, MSI: microsatellite instability, CI: confidence interval

Table 3. Relationship between CD3 and CD8 densities with differentiation, location, depth of invasion, lymph node involvement, TB and MMR status

	CD3 low	CD3 high	Total (%)	p (95%CI)	CD8 low	CD8 high	Total (%)	p (95%CI)
Differentiation								
Poor	3	2	5 (7.4)	0.349	2	3	5 (7.4)	1
Well	22	41	63 (92.6)	(0.434-18.005)	21	42	63 (92.6)	(0.207-8.602)
Location								
Right	4	3	7 (10.3)	0.409	3	4	7 (10.3)	0.681
Left	21	40	61(89.7)	(0.519-12.422)	20	41	61 (89.7)	(0.314-7.536)
Depth of invasion								
pT3-pT4	18	33	51 (75)	0.885	18	33	51 (75)	0.882
pT1-pT2	7	10	17 (25)	(0.253-2.397)	5	12	17 (25)	(0.398-4.307)
Lymph node								
Yes	13	24	37 (54.4)	0.959	13	24	37 (54.4)	1
No	12	19	31 (45.6)	(0.319-2.305)	10	21	31 (45.6)	(0.414-3.127)
Tumor budding								
≥5	15	27	42 (61.8)	1	16	26	42 (61.8)	0.495
0-4	10	16	26 (38.2)	(0.323-2.444)	7	19	26 (38.2)	(0.575-4.856)
Microsatellite status								
pMMR	21	32	53 (77.9)	0.538	20	33	53 (77.9)	0.331
dMMR	4	11	15 (22.1)	(0.507-6.425)	3	12	15 (22.1)	(0.609-9.652)

between MMR status with the depth of invasion and lymph node involvement.[21]

In our study, we found no relationship between TB with tumor differentiation, location, and MMR status. We found 7 cases with TB 0 (10.3%), with good differentiation. Our finding is also similar to Ezenkwa, who reported that the absence or presence of TB depends on the level of differentiation. They found that TB was absent in well-differentiated tumor, but more frequent in poorly differentiated tumors.[22] A study by Romiti found that there was no association between TB with tumor differentiation, location, and MMR status.[23] Our study only found 5 poor differentiation cases, so it is not possible to analysis statistically. Analysis between TB and location show no statistically significant result. This is probably because only 8 cases (11.8%) of our samples were from right-sided locations. Similarly with a study by Archilla, also found that no association between TB and tumor location, but most stage I had low TB.[24] In our study, a significant relationship between TB with the depth of invasion and lymph node involvement was found. Similarly results also found by Fujiyoshi that the level of TB is related to the depth of tumor invasion, the number of positive lymph nodes, the stage of the disease according to the AJCC, and poor tumor differentiation. High TB values are associated with independent tumor molecular features, disease stage, differentiation level, lymphocyte reaction and cytotoxic T cell density.[2] Two other study were found that higher TB was associated with a higher pT stage with right tumor location and pN stage.[25,26] Another study by Dhuhani showed a significant relationship between TB with tumor differentiation, location, the depth of invasion and lymph node metastases, but not associated with distant organ metastases.[12] Hibertina also proved that high-grade TB was significantly associated with lymph node metastasis.[14] Contrary to our results Karlberg M found more high-grade TB in the dMMR group with metastases.[19]

We found no significant relationship between CD3 and CD8 T cell densities with tumor differentiation, location, depth of invasion, lymph node involvement, TB, and MMR status. Lea D found that CD3 in IM and CD8 in CT and IM higher in MSI tumors, compare to MSS tumors. They found a strong association between Immunocore with TNM stage and MSI, but no significant association between Immunocore with tumor location, N status, and TB.[27]

Although there are standards for reporting TB, not all cancer treatment centers including those in Indonesia implement TB reporting. TB

itself is considered a parameter for metastasis, so future pathological reports should include TB status. This will be useful for clinicians to be able to determine the best treatment options for patients and whether the patient tends to experience metastases in the near future. Examination of immune cells, in this case T cell density, is not yet commonly carried out in Indonesia due to the very high cost, but it must be considered as one of the routine examinations that must be carried out.

CONCLUSION

Tumor budding plays an important role in the depth of invasion and lymph node involvement in CRC, but there was no significant relationship with differentiation, location, and MMR status. On the other hand, MMR status also had no significant association with differentiation, location, depth of invasion, and lymph node metastasis. Furthermore, no significant relationship between CD3 and CD8 densities with differentiation, location, depth of invasion, lymph node involvement, TB, and MMR status. As an important aspect of CRC tumor development, reporting TB by the pathologist can help clinicians in their decision to treat CRC patients.

ACKNOWLEDGMENTS

The authors would like to thank Ika Dhuhani, MD, Pathologist as the second pathologist, and also to the Medical Record and Pathology Anatomy Department of MRCCC Siloam Semanggi and RSUPN Dr. Cipto Mangunkusumo.

CONFLICT OF INTEREST

All authors have no conflict of interest.

REFERENCES

1. Ferlay J, Ervik M, Lam F, et al. Global cancer observatory: Cancer today lyon. France:: International Agency for Research on Cancer; 2024 [Available from: https://gco.iarc.who.int/media/globocan/factsheets/populations/360-indonesia-fact-sheet.pdf.
2. Fujiyoshi K, Vayrynen JP, Borowsky J, et al. Tumour budding, poorly differentiated clusters, and T-cell response in colorectal cancer. EBioMedicine. 2020;57:102860.
3. Lugli A, Kirsch R, Ajioka Y, et al. Recommendations for reporting tumor budding in colorectal cancer based on the International Tumor Budding Consensus Conference (ITBCC) 2016. Mod Pathol. 2017;30(9):1299-311.
4. Lea D, Haland S, Hagland HR, Soreide K. Accuracy of TNM staging in colorectal cancer: a review of current culprits, the modern role of morphology and stepping-stones for improvements in the molecular era. Scand J Gastroenterol. 2014;49(10):1153-63.
5. Ahmed Farag AF, Elbarmelgi MY, Azim HA, Abozeid AA, Mashhour AN. TNMF versus TNM in staging of colorectal cancer. Int J Surg. 2016;27:147-50.
6. De Smedt L, Palmans S, Sagaert X. Tumour budding in colorectal cancer: what do we know and what can we do? Virchows Arch. 2016;468(4):397-408.
7. Bruni D, Angell HK, Galon J. The immune contexture and Immunoscore in cancer prognosis and therapeutic efficacy. Nat Rev Cancer. 2020;20(11):662-80.
8. De' Angelis GL, Bottarelli L, Azzoni C, et al. Microsatellite instability in colorectal cancer. Acta Biomed. 2018;89(9-S):97-101.
9. Cohen R, Rousseau B, Vidal J, Colle R, Diaz LA, Jr., André T. Immune checkpoint inhibition in colorectal cancer: Microsatellite instability and beyond. Target Oncol. 2020;15(1):11-24.
10. Zhang Y, Zhang F, Zhao L, Fu X, Shang Y, Gao Q. Long-term survival of a patient with microsatellite-stable refractory colorectal cancer with regorafenib and PD-1 inhibitor sintilimab: a case report and review of literature. BMC Gastroenterol. 2021;21(1):399.
11. Marx AH, Mickler C, Sauter G, et al. High-grade intratumoral tumor budding is a predictor for lymphovascular invasion and adverse outcome in stage II colorectal cancer. Int J Colorectal Dis. 2020;35(2):259-68.
12. Dhuhani I, Handjari DR, Rahadiani N, Krisnuhoni E, M S. Relationship between clinicopathological profile and tumor budding status in colorectal adenocarcinoma at Dr. Cipto Mangunkusumo General National Hospital: A retrospective study. The Indonesian Journal of Gastroenterology, Hepatology and Digestive Endoscopy. 2022;23:193-9.
13. Jessup JM, Goldberg RM, Asare EA, et al. Colon and rectum. In: Mahul B. Amin M, FCAP, editor. AJCC cancer staging manual. 8 ed. American College of Surgeons, 633 N Saint Clair St, Chicago, IL 60611-3211 USA: Springer; 2018. p. 251-74.
14. Hilbertina N, Siregar NC, Abdullah M, SI W. Determination of cancer-associated fibroblast and stromal phenotype as novel prognostic factors for colorectal carcinomas associated with tumor budding. J Nat Sc Biol Med. 2019;10:S68-72.
15. Kwak Y, Koh J, Kim DW, Kang SB, Kim WH, Lee HS. Immunoscore encompassing CD3+ and CD8+ T cell densities in distant metastasis is a robust prognostic marker for advanced colorectal cancer. Oncotarget.

2016;7(49):81778-90.
16. Bankhead P, Loughrey MB, Fernandez JA, et al. QuPath: Open source software for digital pathology image analysis. Sci Rep. 2017;7(1):16878.
17. Remo A, Fassan M, Lanza G. Immunohistochemical evaluation of mismatch repair proteins in colorectal carcinoma: the AIFEG/GIPAD proposal. Pathologica. 2016;108(3):104-9.
18. Jang MH, Kim S, Hwang DY, et al. BRAF-mutated colorectal cancer exhibits distinct clinicopathological features from wild-type BRAF-expressing cancer independent of the microsatellite instability status. J Korean Med Sci. 2017;32(1):38-46.
19. Karlberg M, Stenstedt K, Hallstrom M, Ragnhammar P, Lenander C, Edler D. Tumor budding versus mismatch repair status in colorectal cancer - An exploratory analysis. Anticancer Res. 2018;38(8):4713-21.
20. Andersen HS, Bertelsen CA, Henriksen R, et al. The pathological phenotype of colon cancer with microsatellite instability. Dan Med J. 2016;63(2).
21. Topal U, Gulcan P, Yuksel S, Teke Z, Bektas H, Duman M. The relationship between microsatellite instability in colorectal adenocarcinoma and tumor budding and histopathological parameters. Eur Rev Med Pharmacol Sci. 2023;27(20):9793-800.
22. Ezenkwa US, Ogun GO, Bamiro OO, et al. Pattern and Significance of Tumour Budding in Colorectal Carcinomas Using ITBCC Guidelines: a Low Resource Setting Practice Observation. J Gastrointest Cancer. 2020;51(3):1018-23.
23. Romiti A RM, Marchetti P, Di Cerbo A, Falcone R, Campisi G, Ferri M, Balducci G, Ramacciato G, Ruco L, Pilozzi E. Study of histopathologic parameters to define the prognosis of stage II colon cancer. International Journal of Colorectal Disease. 2019.
24. Archilla I, Diaz-Mercedes S, Aguirre JJ, et al. Lymph node tumor burden correlates with tumor budding and poorly differentiated clusters: A new prognostic factor in colorectal carcinoma? Clin Transl Gastroenterol. 2021;12(3):e00303.
25. Sogutcu N, Gumus S, Abidin Balkan AZ, Bilge H, Cakabay B. Side specific differences of tumor budding on non-metastatic colon cancer. Eur Rev Med Pharmacol Sci. 2022;26(20):7649-55.
26. Zlobec I, Dawson HE, Blank A, et al. Are tumour grade and tumour budding equivalent in colorectal cancer? A retrospective analysis of 771 patients. Eur J Cancer. 2020;130:139-45.
27. Lea D, Watson M, Skaland I, et al. A template to quantify the location and density of CD3 + and CD8 + tumor-infiltrating lymphocytes in colon cancer by digital pathology on whole slides for an objective, standardized immune score assessment. Cancer Immunol Immunother. 2021;70(7):2049-57.