P-ISSN 0215-7284
e-ISSN 25279106
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Comparison of Peritumoral Budding Features in Colorectal Adenocarcinoma NOS by Hematoxylin-Eosin (HE) Staining and Pancytokeratin (AE1/AE3) Immunohistochemistry

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Received : 02-08-2023
Accepted : 07-09-2023
Published : 31-05-2025

ABSTRACT

Background
Colorectal adenocarcinoma is defined as a malignant epithelial tumor of the large intestine (colon and rectum) that shows glandular and mucinous differentiation, accompanied by invasion through the muscularis mucosae into the submucosal layer. Peritumoral budding refers to tumor budding at the leading edge of the tumor and can be considered as one of the prognostic factors. Immunohistochemistry Pancytokeratin (AE1/AE3) is observed in the epithelium, and most carcinomas (tumors originating from epithelial cells) are stained in the cytoplasm.

Methods
The analytical study involved 48 paraffin block samples diagnosed as colorectal adenocarcinoma NOS at Haji Adam Malik Central General Hospital in Medan and the laboratory of the Faculty of Medicine, Universitas Sumatera Utara. The assessment of tumor budding using hematoxylin-eosin staining and pancytokeratin (AE1/AE3) immunohistochemical staining was classified equally into three categories: low budding category if 0-4 buds of tumor budding were observed, intermediate budding category if 5-9 buds of tumor budding were observed, and high budding category if ≥10 buds of tumor budding were observed.

Results
There is no difference in assessing peritumoral budding using Hematoxylin-Eosin (HE) staining and pancytokeratin (AE1/AE3) immunohistochemical staining.

Conclusion
Assessment of peritumoral budding is recommended using Hematoxylin-Eosin (HE) staining.

Keywords: Colorectal adenocarcinoma NOS, peritumoral budding, HE, AE1/AE3

Maj Patol Indones 2025; 34(2): 922-929
DOI: 10.55816/mpi.v34i2.668

P-ISSN 0215-7284
e-ISSN 25279106
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INTRODUCTION

Colorectal adenocarcinoma is one of the most commonly encountered types of colorectal cancer (CRC). Colorectal adenocarcinoma is defined as a malignant epithelial tumor in the large intestine (colon and rectum) that exhibits glandular and mucinous differentiation, accompanied by invasion through the muscularis mucosa into the submucosal layer.[1] CRC is one of the most common malignancy and a leading cause of cancer-related deaths worldwide. According to the Global Burden of Cancer (GLOBOCAN) in 2020, CRC ranked third as the most prevalent cancer after breast cancer and lung cancer.[2]

Tumor budding is defined as the presence of single cells or small clusters of up to four cells at the invasive margin of CRC that exhibit poor differentiation within the tumor stroma, primarily located at the leading edge of the invasive tumor. Tumor budding is categorized into peritumoral budding (PTB), which refers to tumor budding at the leading edge of the tumor, and intratumoral budding (ITB), which represents tumor budding within the main tumor mass.[3-6]

Tumor budding has been shown in various studies to be associated with poor clinical outcomes in various subgroups of CRC. High grade tumor budding is associated with a poor prognosis, the group of poorly differentiated cells is a group of ≥5 tumor cells, without gland formation. However, in evaluating the tumor budding assessment, there is still much controversy in the scoring system, including in the staining of preparations. Tumor budding in CRC can be assessed by Hematoxylin-Eosin (HE) staining, but the heavy inflammatory infiltrate at the front of the invasive tumor makes it difficult to distinguish between budding tumors from lymphocytes, histiocytes and reactive stromal cells. According to the 2016 International Tumor Budding Consensus Conference (ITBCC), pancytokeratin immunohistochemical examination (AE1/AE3) can help pathologists evaluate this problem. According to Koelzer et al,. The use of pancytokeratin for tumor budding assessment has several advantages over HE staining. First, staining with pancytokeratin immunohistochemistry provides three to six times more yield than HE staining, better depicts tumor biology at the invasion front and misses less tumor budding in areas of dense peritumoral inflammation and can differentiate from fibroblasts well. Second, pathologists who are less experienced with tumor budding feel more confident with immunohistochemical staining. Objective reliability is also reflected in the greater inter-observer agreement achieved with pancytokeratin than with HE.[3,7]

METHODS

This study is an analytic study with a cross sectional approach. The research was conducted at the Anatomical Pathology Laboratory of the Faculty of Medicine of Universitas Sumatera Utara and the Haji Adam Malik Central General Hospital Medan from December 2022 to May 2023. The population in this study were patients with colorectal adenocarcinoma NOS who underwent colectomy surgery and their tissue samples were examined for histopathological diagnosis with HE staining. The sample size in this study was calculated by looking at the proportion of incidence of colorectal adenocarcinoma NOS cases in Indonesia sourced from GLOBOCAN Indonesia in 2020 of 8.6%.[1]

The assessment of tumor budding in this study was based on the International Tumor Budding Consensus Conference (ITBCC) in 2016, which recommended criteria for evaluating tumor budding. Firstly, tumor budding was defined as single tumor cells or clusters of ≤4 tumor cells. Secondly, tumor budding should be assessed at the invasive tumor front within a measurement field of 0.785 mm^2, corresponding to a 20 times objective lens. The assessment of tumor budding using both HE staining and immuno-histochemical staining with pancytokeratin (AE1/AE3), was classified equally into the categories of low budding if 0-4 buds were observed, intermediate budding if 5-9 buds were observed, and high budding if ≥10 buds were observed.[6]

Immunohistochemical (IHC) staining with pancytokeratin involves the use of a rabbit polyclonal antibody (Diagnostic BioSystems, Netherlands) at a dilution of 1:100, with an incubation time of 30 minutes at room temperature. Cells that exhibit positive staining are indicated by brown staining in the cytoplasm. Positive controls were obtained from colorectal adenocarcinoma NOS tissue.[8]

RESULTS

In this study, a total of 48 samples diagnosed as colorectal adenocarcinoma NOS, were obtained from colectomy procedures at the Haji Adam Malik Central General Hospital Medan's Department of Anatomic Pathology. The study was aimed to assess the comparison between peritumoral budding using HE staining and Pancytokeratin (AE1/AE3) immunohisto-

Maj Patol Indones 2025; 34(2): 922-929
DOI: 10.55816/mpi.v34i2.668

P-ISSN 0215-7284
e-ISSN 25279106
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chemistry. Table 1 shows the frequency distribution of age, gender, tumor location, histopathological grading, stromal tumor infiltrating lymphocytes (sTILs), vascular invasion, perineural invasion, lymphatic invasion, lymph node involvement, depth of invasion, and peritumoral budding using HE staining. Peritumoral budding was also stained using immunohistochemistry with Pancytokeratin (AE1/AE3).

Table 1. Frequency distribution table of colorectal adenocarcinoma NOS based on clinicopathology.

Variable	N=48	%
Age		
≤30 years old	2	4.2
31-40 years old	7	14.6
41-50 years old	9	18.7
51-60 years old	18	37.5
61-70 years old	8	16.7
71-80 years old	3	6.2
>80 years old	1	2.1
Gender		
Men	27	56.3
Women	21	43.7
Site		
Right colon	12	25.0
Left colon	22	45.8
Rectum	14	29.2
Grading histopathology		
Low grade	37	77.1
High grade	11	22.9
Stromal TILs		
Poor	31	64.6
Rich	17	35.4
Vascular invasion		
None	20	41.7
IMVI	6	12.5
EMVI	18	37.5
IMVI dan EMVI	4	8.3
Perineural invasion		
No	35	72.9
Yes	13	21.1
Limfatic invasion		
No	30	62.5
Yes	18	37.5
Staging		
T1	1	2.1
T2	8	16.7
T3	36	75.0
T4	3	6.2
Lymph node		
NO	40	83.3
N1	8	16.7
Peritumoral budding (HE)		
Low budding	24	50.0
Intermediate budding	15	31.2
High budding	9	18.8
Peritumoral budding (AE1/AE3)		
Low budding	22	45.8
Intermediate budding	15	31.3
High budding	11	22.9

In this age category, the data was obtained from medical records, and the highest number of cases of colorectal adenocarcinoma NOS occurred in the age group of 51-60 years old, with a total of 18 (37.5%) samples. The youngest patient was 22 years old, and the oldest patient was 83 years old. The second most common age group was found in the 41-50 years old range, with a total of 9 (18.7%) samples, followed by the third age group of 61-70 years old, with 8 (16.7%) samples. The next age group, 31-40 years old, had 7 (14.6%) samples, and the age group of 71-80 had 3 samples. The age group ≤30 years old ranked second to last with 2 (4.2%) samples, and the least common age group was >80 years old, with only 1 (2.1%) sample. In terms of gender, the data was also obtained from medical records, and it was found that there were more male patients, with a total of 27 (56.3%) samples, compared to female patients, with 21 (43.7%) samples. Regarding the tumor location, the data was obtained from medical records as well. The highest number of cases was found in the left colon, with a total of 22 (45.8%) samples, followed by the rectum with 14 (29.2%) samples, and the least common location was the right colon with 12 (25.0%) samples.

Based on the evaluation results, the majority of histopathological grading was low grade, with 37 (77.1%) samples, while high grade accounted for 11 (22.9%) samples. The majority of Stromal TILs were classified as TILs poor, with 35 (64.6%) samples, compared to TILs rich with 17 (35.4%) samples. In terms of vascular invasion, the highest number was found to be no vascular invasion, with 20 (41.7%) samples, while the cases with invasion were divided into two categories: IMVI with 6 (12.5%) samples and EMVI with 18 (37.5%) samples. For perineural invasion, the most commonly observed was no perineural invasion, with 35 (72.9%) samples, while there were 13 (21.1%) samples with perineural invasion. In terms of lymphatic invasion, the majority was found to be no lymphatic invasion, with 30 (62.5%) samples, while there were 18 (37.5%) samples with lymphatic invasion. The most common depth of tumor invasion (T) was T3, with 20 (66.7%) samples, and the least common was T1, with 1 (3.3%) sample. Lymph node involvement was observed in 9 (30%) samples, while 21 (70%) samples had no lymph node involvement. Among those with lymph node involvement, the majority had no lymph node involvement (N0), with 40 (83.3%) samples, while 8 (16.7%) samples had lymph node involvement. Regarding peritumoral budding using HE staining and Pancytokeratin (AE1/AE3) immunohistochemistry, the majority was classified as low budding, with 24 and 22

Maj Patol Indones 2025; 34(2): 922-929
DOI: 10.55816/mpi.v34i2.668

P-ISSN 0215-7284
e-ISSN 25279106
Accredited by KEMENRISTEKDIKTI/Sinta-3

(50.0% and 45.8%) samples, followed by intermediate budding with 15 samples (31.2% and 31.3%), and the least common was high budding, with 9 and 11 (18.8% and 22.9%) samples.

In this study, the results obtained in HE staining with low budding 24 (50.0%) samples, intermediate budding 15 (31.2%) samples and high budding 9 (18.8%) samples, pancytokeratin AE1/AE3 staining obtained low budding 22 (45.8%) samples, intermediate budding 15 (31.3%) samples and high budding 11 (22.9%) samples. To assess the comparison, the Friedman test was performed and the results showed no significant difference in evaluating peritumoral budding using both HE staining and Pancytokeratin AE1/AE3 immunohistochemistry.

Table 2 shows the comparison of peritumoral budding with HE and pancytokeratin (AE1/AE3) staining in colorectal adenocarcinoma NOS using the Friedman test.

Table 2. Comparison table of peritumoral budding assessment by HE staining and pancytokeratin (AE1/AE3) in colorectal adenocarcinoma NOS.

Variable	Pancytokeratin AE1/AE3						p-value
	Low budding		Intermediate budding		High buddding		
	n	%	N	%	n	%	
HE							
Low budding	22	91.7	2	8.3	0	0	0.003*
Intermediate budding	0	0	13	86.7	2	13.3	
High budding	0	0	0	0	9	100	

*Friedman

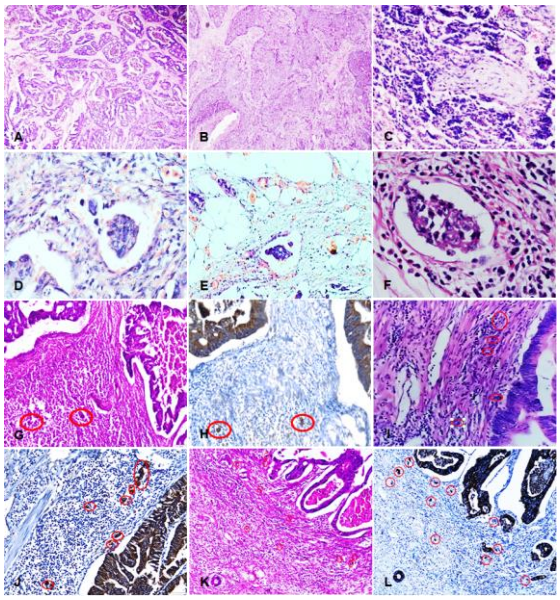

Figure 1. A. Low grade of histopathological grading in colorectal adenocarcinoma (HE, 100 times). B. High grade (HE, 100 times). C. Perineural Invasion (HE, 100 times). D. Intramural vascular invasion (HE, 100 times). E. Extramural vascular invasion (HE, 100 times). F. Lymphatic invasion (HE, 100 times). G. Low Budding (HE, 200 times). H. Low Budding (AE1/AE3, 200 times). I. Intermediate Budding (HE, 200 times). J. Intermediate budding (AE1/AE3, 200 times). K. High Budding (HE, 200 times). L.High Budding (AE1/AE3, 200 times).

In this study, the results of HE staining showed low budding in 24 (50.0%) samples, intermediate budding 15 (31.2%) samples and high budding 9 (18.8%) samples, pancyto-keratin AE1/AE3 staining obtained low budding 22 (45.8%) samples, intermediate budding 15 (31.3%) samples and high budding 11 (22.9%) samples. To assess comparisons, the Friedman test was carried out. This test is used as an alternative when two-way ANOVA in parametric statistics cannot be used because the assumptions required in two-way ANOVA are not fulfilled. And the results are significant with a p-value of 0.003 where the p-value limit is 0.005, which means there is no difference in evaluating peritumoral growth between HE staining and Pancytokeratin AE1/AE3 immunohistochemistry.

DISCUSSION

In this study, 48 samples were obtained from medical records data, with a mean age of 51.6 years old for patients with colorectal adenocarcinoma NOS. The youngest patient was 22 years old, and the oldest was 83 years old. The most common age group among patients was 51-60 years old, with 18 (37.5%) samples. These findings are consistent with previous studies. According to Nasution's study in 2017, the majority of CRC patients were above 50 years old.[9] In 2014, Park et al also found that the majority of patients were 50 years old and older, with a mean age of 60.9 years old.[10] According to the WHO's fifth edition published in 2019, most CRC patients are elderly, and several studies have shown an increased incidence of CRC with advancing age.[1] As age increases, the tissue's ability to repair and replace cells gradually decreases, as well as its ability to maintain normal function. This leads to a weakened immune defense against infections and impaired repair

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P-ISSN 0215-7284
e-ISSN 25279106
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mechanisms, resulting in cumulative changes that reduce the body's resilience and responsiveness to internal and external stimuli, ultimately reducing its ability to combat cancerous cell growth.[11] In patients with CRC under the age of 40, there is typically a family history of conditions such as hereditary non polyposis colorectal cancer (HNPCC), familial adenomatous polyp (FAP), Crohn's disease, and ulcerative colitis.[1,12]

In this study, out of 48 samples, 27 (56.3%) samples of colorectal adenocarcinoma NOS were found to be male, slightly more than female. These findings are consistent with previous studies. Nasution's study in 2017 reported that CRC patients were more common in males, with 44 out of 81 (54.3%) samples.[9] Kwon et al in 2013 also reported that males were more frequently affected by CRC, with 132 out of 256 (51.6%) samples.[13] Mechanisms related to gender differences in CRC occurrence include hormonal differences between males and females. The estrogen receptor ERβ is a protective factor against CRC. Experiments on mice have shown that ERβ increases proliferation and reduces differentiation and apoptosis of colonic mucosal cells. Estrogen can also prevent CRC by reducing inflammation through the inhibition of the inflammatory factor IL-6, which is involved in inflammatory bowel disease (IBD), a known risk factor for CRC.[14] Other studies have suggested that progesterone hormone may also reduce the risk of CRC in women due to its activity in synthesizing endogenous sex hormones. Additionally, unhealthy lifestyle factors in men, such as excessive alcohol consumption, can contribute to their vulnerability to this malignancy. Excessive alcohol consumption alters the normal state of the digestive tract mucosa. This is caused by the oxidation of acetaldehyde, a product of ethanol metabolism, which promotes inflammation in the digestive tract mucosa and abnormal cell growth. Acetaldehyde also disrupts DNA repair processes by inhibiting relevant enzymes. Furthermore, acetaldehyde can bind to other molecules, leading to DNA mutations that trigger carcinogenesis.[15]

The most common location for colorectal adenocarcinoma NOS in this study was the left colon, with 22 (45.8%) samples. The next most common location was the rectum, with 14 (29.2%) samples, followed by the right colon, which had the fewest cases at 12 (25.0%) samples. These findings are consistent with previous studies. Park et al reported that the majority of CRC cases were located in the left colon, accounting for 451 out of 579 (77.9%) cases.[9] Wang et al also reported that the left colon was the most frequent site, with 15,880 out of 26,908 (67.7%) cases.[16] Symptoms of CRC in the right colon often go unnoticed, unlike the left colon and rectum, which commonly present symptoms such as pain, constipation, and bloody stools. This can be attributed to the larger tumor diameter and different growth patterns. In the right-sided colon, the tumor can grow to a larger size while still being clinically asymptomatic and take longer to manifest symptoms.[1,17]

The histopathological grading of colorectal adenocarcinoma is determined based on the percentage of glandular differentiation components. According to the WHO 2019 classification, this grading is divided into two categories: low grade and high grade. In this study, the majority of cases were classified as low grade, with 37 (77.1%) samples, followed by high grade with 11 (22.9%) samples. These findings are consistent with previous studies. Schwarz et al in 2019 found that low-grade cases were the most common in CRC, accounting for 576 out of 180 (73.6%) cases.[18] Zlobec et al in 2020 reported that low-grade cases accounted for 81.9% of 771 cases of CRC.[19] This could be attributed to the fact that many samples in this study were located in the left colon, leading to prompt medical evaluation through colonoscopy or other imaging techniques, which facilitated the early detection of cancer.

In this study, the highest number of stromal TILs was found in the stromal TILs poor category, with 31 (64.6%) samples, while 17 (35.4%) samples belonged to the stromal TILs rich category. These findings are consistent with the study by Fard et al in 2019, which reported 78 (84.8%) samples in the TILs poor category and 14 (15.2%) samples in the TILs rich category. Many studies have reported the role of histological assessment of TILs in predicting MSI status in CRC. Specifically, TILs are associated with host immunity status, and various reports have demonstrated that TILs levels serve as beneficial biomarkers in the prognosis of various malignancies, including CRC.[20]

In this study, vascular invasion was divided into intramural vascular invasion (IMVI) and extramural vascular invasion (EMVI). From the evaluation results, it was observed that histopathological examination revealed a higher prevalence of EMVI, with 18 (37.5%) samples, compared to IMVI, with 6 (12.5%) samples. This finding is consistent with the

Maj Patol Indones 2025; 34(2): 922-929
DOI: 10.55816/mpi.v34i2.668

P-ISSN 0215-7284
e-ISSN 25279106
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literature, as stated in the WHO 2019 book, which indicates a higher incidence of EMVI compared to IMVI, and is also in line with previous studies. Bedge et al found an incidence of 16% for IMVI and 62% for EMVI out of 87 cases of vascular invasion.[21] Gibson et al reported a higher prevalence of EMVI (15.1%) compared to IMVI (3.3%).[22] It is known that the incidence of EMVI is higher than IMVI, but this observation is still considered underreported. EMVI is known as an independent predictor of poor prognosis after resection in CRC, while the role of IMVI is not yet well understood. The negative prognostic impact of EMVI is higher than that of IMVI.[1,22]

In this study, 13 (21.1%) samples showed perineural invasion, which is not significantly different from the incidence mentioned in the WHO 2019 book on GIT, stating that the incidence of perineural invasion is approximately 20%.[2] This finding is also consistent with previous studies. Knijn et al reported a perineural invasion rate of 24.3% out of 7653 cases in their meta-analysis study in 2016.[23] Perineural invasion indicates a significantly lower 5-year survival rate and signifies more advanced disease.[23] Perineural invasion status has been reported as a complementary factor for TNM staging in CRC. Zhou et al found in their study that perineural invasion status has a significant impact on the overall survival of patients with stage II and III CRC. Patients in stage II are the most important population to benefit from the identification of perineural invasion, as they can be considered for adjuvant chemotherapy when perineural invasion status is known.[24]

In this study, lymphatic invasion was found in 18 (37.5%) samples. This result is slightly higher compared to the study by Betge et al who found lymphatic invasion in 126 (33%) samples.[21] Lymphatic invasion is associated with lymph node metastasis and is a poor prognostic indicator in CRC patients, as it is related to lower survival rates compared to CRC patients without lymphatic invasion.[1,25]

The most commonly found tumor invasion depth in this study was T3 (invasion of the tumor into the subserosal layer) in 36 (75.0%) samples. This finding is consistent with the study conducted by Betge et al in 2012, which found that T3 invasion depth was the most common, with 218 (57.2%) samples.[21] The depth of tumor invasion and the extent of local resection in CRC are crucial in determining the risk of local recurrence. Invasion depth beyond T1 allows for invasion into vascular and lymphatic structures, as well as distant metastasis. The depth of invasion can also affect the prognosis of CRC.[26]

In this study, lymph node involvement (N) was found in 8 (16.7%) samples, while 40 (83.3%) samples had no lymph node involvement. Lymph node involvement is a poor prognostic indicator in patients with colorectal adenocarcinoma, as it is associated with lower survival rates compared to patients without lymph node involvement.[1,27]

The assessment of peritumoral budding in this study was conducted in front of the invasive tumor after selecting the hotspot with the highest number of buds. Both HE staining and Pancytokeratin (AE1/AE3) were used, and the majority of samples showed low budding in both staining methods. This finding is consistent with a study by van Wyk et al, which found that low budding was more prevalent than high budding, accounting for 72% of cases.[28] High-grade tumor budding has been shown in various studies to be associated with poor clinical outcomes in different subsets of colorectal cancer (CRC). In CRC, tumor budding is associated with an increased risk of lymph node metastasis and reduced survival rates and recurrence.[28]

The relationship between the variables used in this study was tested using the Friedman test, and the results showed significant, which means there was no difference in assessing peritumoral budding using either HE staining or immunohisto-chemistry with pancytokeratin (AE1/AE3). This finding is consistent with a study by Pujawan et al, which stated that statistical analysis using the McNemar test showed no difference in the grading of tumor budding in colorectal adenocarcinoma NOS diagnosed with HE staining compared to pancytokeratin staining.[29] According to the authors, although there may be differences in the number of peritumoral budding assessments between HE staining and immunohistochemistry with pancytokeratin (AE1/AE3), these differences are not statistically significant. The variations in assessing peritumoral budding are attributed to reactive stromal cells and extensive inflammatory cells, and immunohistochemistry with pancytokeratin (AE1/AE3) can assist in evaluating peritumoral budding under such conditions.

CONCLUSION

The majority of patients with colorectal adenocarcinoma NOS are in the age range of 51-60 years old, with a mean age of 51.6 years old. The youngest patient was 22 years old,

Maj Patol Indones 2025; 34(2): 922-929
DOI: 10.55816/mpi.v34i2.668

P-ISSN 0215-7284
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while the oldest was 83 years old. There is a higher prevalence of colorectal adenocarcinoma NOS in males compared to females. The most common location is the left colon. Low-grade histopathological grading is more prevalent than high-grade grading. Stromal TILs are predominantly classified as poor, rather than rich. The majority of cases do not exhibit vascular invasion, lymphatic invasion, perineural invasion, or lymph node metastasis. The most common depth of tumor invasion is T3. Peritumoral budding analysis using both HE staining and pancytokeratin (AE1/AE3) reveals a higher prevalence of low-grade budding. There is no significant difference in assessing peritumoral budding between HE staining and immunohistochemistry with pancytokeratin (AE1/AE3).

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