

TUMOR BUDDING AS A PROGNOSTIC MARKER IN PRIMARY COLON CANCER – A SINGLE CENTER EXPERIENCE

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ABSTRACT

Introduction: Tumor budding (TB) is considered to be a morphological and prognostic factor relevant to colon cancer (CC). The aim of our study is to assess the TB and to evaluate its relationship to clinicopathological findings within stage II and III CC patients as a single center experience.

Materials and methods: A total of 120 CC patients operated between 2018 and 2021 at the University Clinic of Digestive Surgery in Skopje, the Republic of North Macedonia were included in this retrospective, single center study. TB was evaluated by the magnification of 200x along the invasive front of the primary tumor on H&E and CKAE1/AE3 immunohistochemically stained sections. Two grades were used: low grade (TB1, 0-4 TBs) and high-grade, which includes intermediate (TB2, 5-9 TBs) and high grade (TB3 $\geq$ 10TBs) of TBs.

Results: A statistically significant correlation has been identified between high-grade TB and age ($p=0.05$) of the patients. There was also a significantly higher occurrence of high-grade TB in patients within stage III CC. Statistically significant correlations were also found in lymph node status ($p<0.01$), vascular invasion ($p<0.05$), lymphatic invasion ($p<0.01$), postoperative relapse ($p<0.01$), and death ($p<0.01$). Tumor relapse and death were significantly more frequent in patients with high-grade TB than those with low-grade TB. Patients with registered high-grade TB demonstrated significantly lower relapse-free survival (RFS) and overall survival (OS) rates than patients with low-grade TB over the observation period (RFS: 53.8% vs. 98.5%, $p<0.001$; OS: 65.4% vs. 97.1%, $p<0.001$, respectively). Patients with lung and liver postoperative relapses had higher percentage of cases with high-grade TB (94.1%).

Conclusion: Our results are highly suggestive that TB should be included as a histological biomarker in the pathology report of patients with stage II and stage III CC, because of its prognostic value.

Keywords: colon cancer, tumor budding, survival, relapse, prognostic marker

INTRODUCTION

Colon cancer (CC) is one of the most common malignant tumors in the Western World, and it is typically considered a major public health concern [1, 2]. According to Global Cancer Statistics 2020, CC is the third most common cancer in the world, and the second leading cause of death after lung cancer in men and breast cancer in women [2]. The development of CC is the result of a continuous, long-term, and multifactorial process that includes various genetic predispositions, environmental exposure factors and inflammatory conditions of the digestive tract [3].

In clinical practice, the gold standard for the staging of malignant tumors is the tumor–node–metastasis (TNM) staging system, which was published simultaneously by the Union for International Cancer Control (UICC) and by the American Joint Committee on Cancer (AJCC). Both the TNM staging system and the well-established tumor-related prognostic factors such as lympho-vascular and perineural invasion are utilized to assess the risk of recurrence, and the recommendation process of adjuvant chemotherapy for patients with high-risk stage II and stage III CC [4, 7].

The current treatments for CC involve multiple modalities such as surgery, chemotherapy, target immunotherapy, and other palliative treatments [4, 5]. Curative surgical resection is a crucial treatment for patients with stage I and II CC, whereas, for stage III the standard treatment is a combination of surgical resection and adjuvant chemotherapy. In patients with stage III CC, the current standard of care is an adjuvant chemotherapy that combines fluoropyrimidine (FOLFOX and FOLFIRI) and oxaliplatin (CAPOX) [6].

Research efforts in the field of CC persistently focus on finding prognostic and predictive markers that facilitate the optimization of the treatment decision-making process, and thus improve overall survival (OS). A major challenge is to identify patients at risk of developing future distant metastases following their initial diagnosis and curative surgery. This holds particular significance for patients diagnosed with stage II CRC. While the majority achieve a cure through surgical resection, approximately 20-25% of them will develop metastases within a 5-year period [5, 8].

Tumor budding (TB) is considered to be a morphological and prognostic factor relevant to CC. By definition, TB represents a single tumor epithelial cell or a cluster of up to four cells at the invasive front of the tumor. Higher grades of TB are associated with a higher recurrence rate and a worse OS regardless of the primary pathological stage [9-11]. The standardized scoring systems for TB in CC have been assessed by the International Tumor Budding Consensus Conference (ITBCC) [12]. In stage II CC, high-grade tumor buds (BD3) represent a poor prognostic factor that is considered for guidance of adjuvant treatment decision [13].

The aim of our study is to assess the TB and to evaluate its relationship to the clinicopathological findings within stage II and III CC patients as a single center experience. The results from this evaluation are expected to support the idea of research and establishment of new prognostic factor that would better define the prognosis of the patients with CC.

MATERIALS AND METHODS

Study design

A total of 120 CC patients operated between 2018 and 2021 at the University Clinic of Digestive Surgery in Skopje, Republic of North Macedonia were included in this retrospective, single center study. Resection material of all patients was analyzed at the Institute of Pathology, Medical Faculty, University “Ss. Cyril and Methodius” in Skopje. The clinicopathological data of the included patients were collected from the electronic database containing their medical records and pathology reports. Additional information on postoperative follow-up of the patients including data concerning postoperative relapse were provided by the University Clinic for Radiotherapy and Oncology in Skopje.

Inclusion criteria consist a histologically proven CC, complete curative resection (R0), clinical stage II (T3-4, N0, M0) or III (any T, N1-2, M0) and age over 18 years. Exclusion criteria were as follows: patients with rectal cancer, patients who received neo-adjuvant therapy, a previously diagnosed malignancy within 10 years prior to the current CC, multiple synchronous colon tumors, and a stage I or IV disease. Patients with an insufficient observation period (less than 6 months)

and the deceased within 90 days after the surgical resection were also excluded.

The application of adjuvant chemotherapy after surgery was performed according to the ESMO guidelines [6]. All data and patient material were handled in accordance with the Helsinki Declaration (2013).

Laboratory methods

All surgical specimens were admitted to the Institute of Pathology. After 24 hours fixation, on macroscopical dissection of a 5 millimeters thick slices from the macroscopically deepest tumor invasion, as well as all extracted mesocolic lymph nodes were taken and paraffin embedded. Histological analyses were conducted on 4 to 6 μ thick sections stained with Hematoxylin and Eosin (H&E). Additionally, for easier detection of TB, immunohistochemical analysis of pan-cytokeratin (DAKO Monoclonal Mouse Anti-Human Cytokeratin, Clone AE1/AE3, dilution 1:100) was applied by the use of PT LINK Immunoperoxidase staining.

Assessment of tumor budding

The assessment of TB was performed by two independent pathologists, observers (GP and

DB), as recommended by the consensus [12] by using a hotspot method [23]. Summarising, the process of counting TB was performed as a complementary method by the magnification of 200x along the invasive front of the primary tumor on H&E and CKAE1/AE3 immunohistochemically stained sections. After determining the “hotspots”, the number of TBs was counted under a 20 $\times$ objective lens where the hotspot showed the most frequent TBs. These processes for TB evaluation comply with the ITBCC criteria [12]. However, due to the small cohort of analysed TB variations only two grades [23] i.e. low grade (TB1, 0-4 TBs) and high-grade including intermediate and high grade of TB (TB2, 5-9 TBs or TB3 $\geq$ 10TBs) were used (Figure 1).

Statistical analysis

The series of categorical variables were presented as a number and associated percentages while the subsequent analysis was completed using the Chi-squared test. The rate of relapse-free survival (RFS) was calculated from the date of surgical intervention to the date of relapse diagnosis. The rate of OS was calculated from the date of surgical intervention to the date of death by any cause. Both the RFS and OS rate were

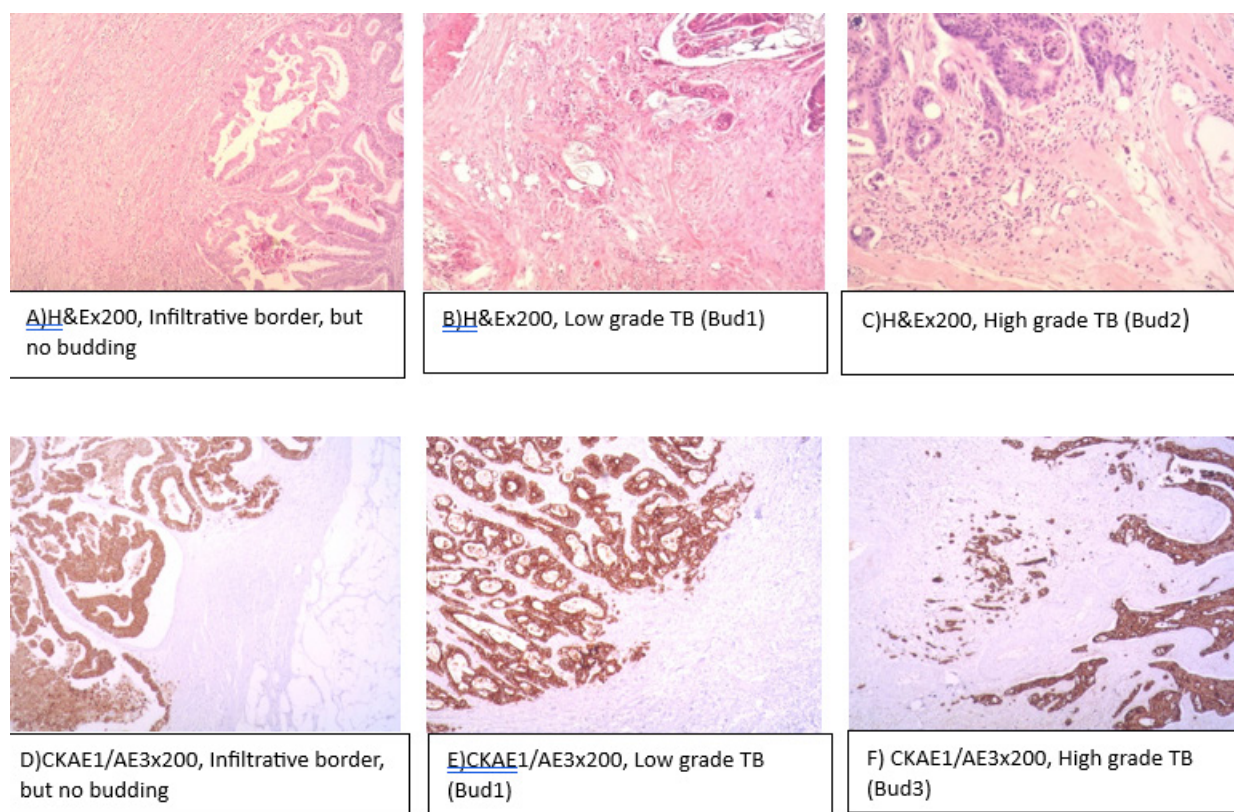


Figure 1. H&E and CKAE1/AE3 stained sections for tumor budding (TB) in CC evaluated according to hotspot method. 200x, NIKON ECLIPSE 80

calculated by applying the Kaplan-Meier method with associated log rank tests. The results were considered to be statistically significant for values where $p < 0.05$. All statistical analyses were calculated by using the statistical software IBM SPSS 29 (Chicago, USA).

RESULTS

Demographic and clinical findings

Patient population characteristics are presented in Table 1. The mean age of the included patients was 64.88 years (SD = 10.18, range = 31-87 years). Males were more frequently affected than females (55% as opposite to 45%, respectively) and the disease was more frequent in the age group between 61 and 70 years. Low-grade TB was found in 68 cases (56.7%), while high-grade TB was found in 52 cases (43.3%). A total of 68 patients (56.7%) received postoperative adjuvant chemotherapy, but two patients (1.7%) stopped after one cycle due to side effects. Postoperative relapse was registered in 25 cases (20.8%), of which 17 (68%) were associated with liver or lung distant metastases. Out of 25 patients with metastatic disease (20.8% of the cohort), 24 had high grade TB (96% of the group). Among the 17 patients with liver or lung distant metastases, 16 had high grade TB (94.1% of the group). During the observation period of 36 months, death by any cause was registered in 20 cases (16.7%), including 14 cases (11.7%) where death was related to the metastatic disease and six cases (5%) whose death was unrelated to CC.

Correlation between TB and patient population characteristics

The correlation between TB and patient population characteristics by using Chi-square test are presented in Table 2 in detail. A statistically significant correlation has been identified between high grade TB and age ($p = 0.05$) of the patients, with significantly higher occurrence of high grade TB in patients older than 61 years of age (41 from total of 52 cases with high TB). There was also a statistically significant correlation between the stage of disease and the tumor budding at the .01 level, which means that there was a significantly higher occurrence of high-grade TB in patients within stage III. Statistically significant correlations were also found in lymph node status

($p < 0.01$), vascular invasion ($p < 0.05$), lymphatic invasion ($p < 0.01$), postoperative relapse ($p < 0.01$), and death ($p < 0.01$). Especially, tumor relapse and death were significantly more frequent in patients with high grade TB than those with low grade TB. In addition, less significant correlation was found between TB and pT ($p = 0.078$).

TB grade impact on relapse free survival and overall survival rates

The RFS and OS rates after 36 months of observation were 79.2% and 83.3% for the entire cohort, respectively. Patients with registered high-grade TB demonstrated significantly lower RFS and OS rates than patients with low-grade TB over the observation period (RFS: 53.8% vs. 98.5%, $p < 0.001$; OS: 65.4% vs. 97.1%, $p < 0.001$, respectively). The Kaplan-Meier survival curves for RFS and OS according to TB grade are shown in Figure 2 and Figure 3.

DISCUSSION

We presented the results that validate the use of TB to predict postoperative tumor relapse and the OS of patients with stage II and III CC. In our study patients with high-grade TB had significantly higher risk of experiencing tumor relapse and death of any cause in comparison to the patients with low-grade TB. High-grade TB has also been linked to patient population characteristics such as older patients, stage of the disease, and nodal metastases status and lympho-vascular invasion. Furthermore, descriptive statistics in our study showed that patients with lung and liver postoperative relapses had higher percentage of cases with high-grade TB (94.1%). In a cohort study, Smit M. A et al. out of 246 cases found that the high grade TB (≥ 10 buds) group showed a significant worse prognosis compared to the low and intermediate grade group. In their study they analyzed the value of the tumor-stroma rotation (TSR) and TB in stage II and stage III CC patient samples and the high-grade TB group was small with 17 cases (7%). We could speculate that this is comparable with our results, despite the fact that it is a study on a larger group of patients [32].

Our findings are consistent with the existing literature data concerning the concept of TB in CC [14-16]. While TB was first described in 1950, it was first used in a study of CC in 1993 when Hase

Table 1. *Patient population characteristics*

Characteristics	Number	Percentage
Age (years)		
Mean (SD, range)	64.88 (10.18, 31-87)	
Age		
≤40 years	1	0.8
41-50 years	11	9.2
51-60 years	20	16.7
61-70 years	54	45.0
71-80 years	25	20.8
≥81 years	9	7.5
Sex		
Male	66	55.0
Female	54	45.0
Tumor localization		
Left	69	57.5
Right	51	42.5
Stage of the disease		
II	70	58.3
III	50	41.7
pT		
2	2	1.7
3	84	70.0
4	34	28.3
pN		
0	70	58.3
1	33	27.5
2	17	14.2
pV		
0	107	89.2
1	13	10.8
pL		
0	59	49.2
1	61	50.8
Grade		
G2/NG2	107	89.2
G3/NG3	13	10.8
Histological type		
Adenocarcinoma	109	90.8
Mucinous adenocarcinoma	11	9.2
Tumor budding		
Low	68	56.7
High	52	43.3
Postoperative relapse / Metastatic disease (n=25)		
Liver and/or lungs	17	68.0
Others	8	32.0
Death of any cause (n=20)		
Metastatic disease	14	70.0
Other causes	6	30.0

Abbreviations: pL - lymphatic invasion; pV - vascular invasion; G2/NG2 - moderately differentiated adenocarcinoma; G3/NG3 - poorly differentiated adenocarcinoma.

Table 2. Relationship between tumor budding and patient population characteristics

Characteristics	Tumor budding		P
	Low	High	
Sex			0.604
Male	36	30	
Female	32	22	
Age (per decade)			0.149
≤40 years	0	1	
41-50 years	6	5	
51-60 years	15	5	
61-70 years	33	21	
71-80 years	11	14	
≥81 years	3	6	
Tumor localization			0.280
Left	42	27	
Right	26	25	
Stage of the disease			<0.0001**
II	54	16	
III	14	36	
pT			0.092
2	1	1	
3	53	31	
4	14	20	
pN			<0.0001**
0	54	16	
1	13	20	
2	1	16	
pV			0.046*
0	64	43	
1	4	9	
pL			<0.0001**
0	45	14	
1	23	38	
Grade			0.333
G2/NG2	59	48	
G3/NG3	9	4	
Histology type			0.259
Adenocarcinoma	60	49	
Mucinous adenocarcinoma	8	3	
Postoperative relapse/ Metastatic disease			<0.0001**
No	67	28	
Yes	1	24	
Postoperative relapse / Metastatic disease by localization			0.484
Liver and/or lungs	1	16	
Others	0	8	
Death of any cause			<0.0001**
No	66	34	
Yes	2	18	

* statistically significant at the .05 level

** statistically significant at the .01 level

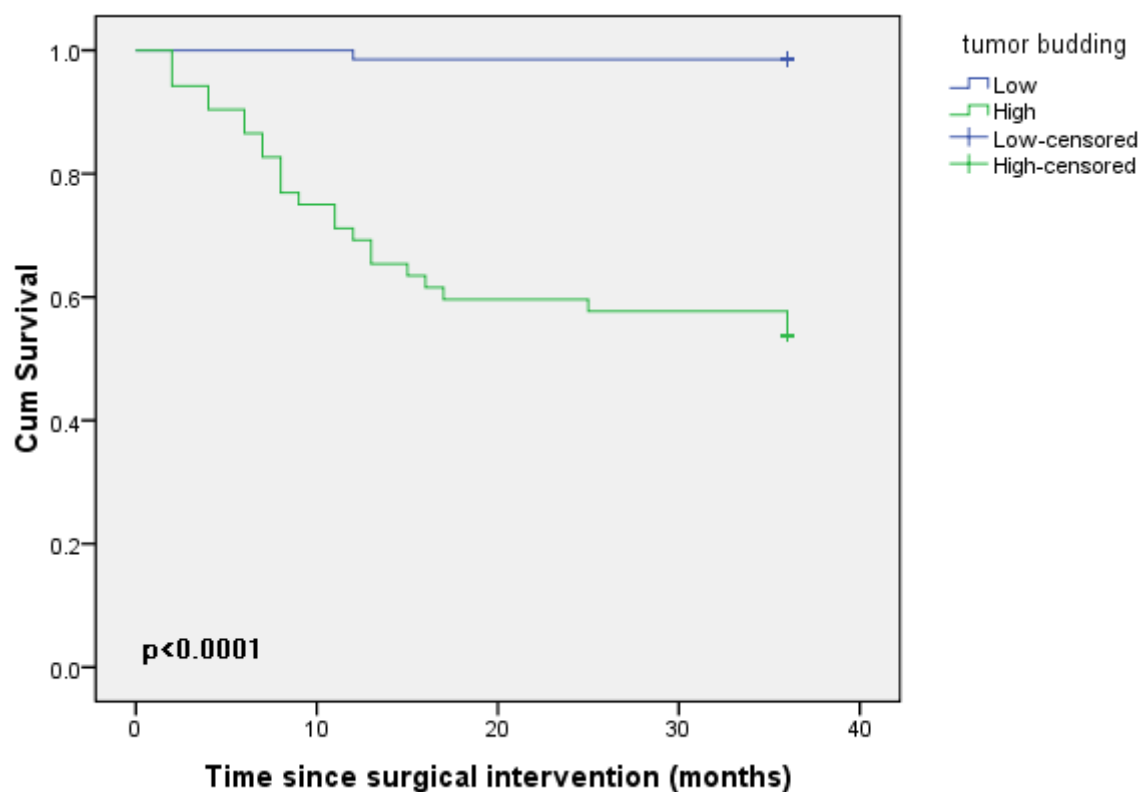


Figure 2. The Kaplan-Meier relapse-free survival (RFS) curves in patients with low- and high-grade TB

Number at risk	Time since surgical intervention (months)							
	0	5	10	15	20	25	30	35
Low-grade TB	68	68	68	67	67	67	67	34
High-grade TB	52	47	39	34	31	31	30	16

et al. showed a significant reduction in 5-year and 10-year OS survival rates in patients with CC who had a high-grade TB [16]. The utility of TB as a histological indicator has also been validated in several types of solid tumors, such as esophageal, pancreatic, mammary, nasopharyngeal, pulmonary, gastric, and endometrioid cancers [17-22].

The results of our study are also consistent with the findings of the recent studies [23, 27]. In a retrospective cohort study of Saito et al., where 213 patients were included, the high-grade TB was associated with the increased presence of lymphatic and venous invasion, as well as with the higher frequency of tumor relapse and death [23]. The authors also concluded that the administration of adjuvant chemotherapy to stage II CC patients with high-grade TB may reduce postoperative relapse among them. Koelzer et al. also demon-

strated that a high degree of TB is correlated with greater values of pT, or presence of lymph node metastasis, lympho-vascular invasion, perineural invasion, distant metastasis, and overall negative survival in stage II CC patients [24].

The application of TB budding as a prognostic factor has both advantages and disadvantages. In general, TB is considered as intensity-dependent prognostic factor, which represents an important clinical advantage [23]. The number of TBs identified can be linked to the relevant risk factors. The disadvantage lies in the fact that the determination of TB can be difficult and masked by several factors, such as the presence of active fibroblasts in desmoplastic stroma, as well as the presence of an intense inflammatory infiltration.

During the microscopy process, TBs were evident as groups of cells that have detached from

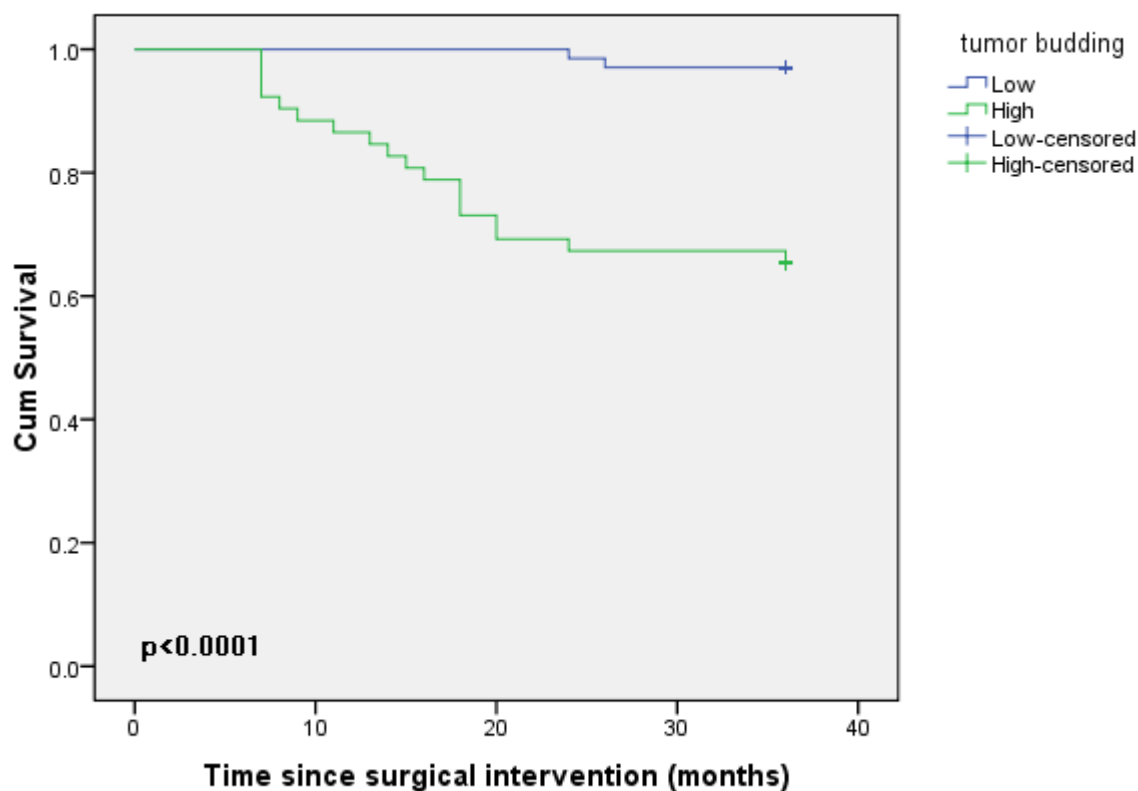


Figure 3. The Kaplan-Meier overall survival (OS) curves in patients with low- and high-grade TB

Number at risk	Time since surgical intervention (months)							
	0	5	10	15	20	25	30	35
Low-grade TB	68	68	68	68	68	67	66	33
High-grade TB	52	52	46	43	38	35	35	18

the primary tumor mass. Through the analysis of consecutive sections from tumors with significant budding, stained with anti-cytokeratin antibodies, Prall et al. established that the majority of these buds appear to represent discrete clusters of cells, which are in fact connected to adjacent larger glands [25]. Several studies have shown that evaluation performed using H&E-stained slides provide lower TB values when compared to the use of immunohistochemical stains [26, 32]. In our study, we examined TB variation using two grades [23] i.e. low grade (TB1, 0-4 TBs) and high grade, including intermediate and high group with TB (TB2, 5-9 TBs and TB3 ≥ 10 TBs) without comparing the results from the H&E evaluation

and immunohistochemical stain of the TB as a complementary method.

We found that the patients with registered high-grade TB demonstrated significantly lower RFS and OS rates than the patients with low-grade TB over the observation period (RFS: 53.8% vs. 98.5%; OS: 65.4% vs. 97.1%, respectively). These results are in line with the study of Basile et al. where by using analysis on scanned H&E tissue sample slides TB has been assessed in three categories: TB 1 (0-4 buds: low), TB2 (5-9 buds: intermediate); and TB3 (≥ 10 buds: high). They found considerably worse 3-year DFS in patients with TB2 and TB3 compared with TB1 patients. However, the same findings were found when patients

from TB2 and TB3 group were placed together in juxtaposed with the TB1 patients. Studies with similar results have been also reported.

There are also studies where only patients with high grade TB with cut of ≥ 10 buds have significant worse prognosis compared to the TB1 and TB2 group of patients [32]. The authors due to a small group with 7% of the cases concluded that TB-high is a prognostic factor, but for a small subgroup of patient population. Limitations of this study that should be considered when interpreting the results are its retrospective design and the relatively small sample of the patients. Furthermore, the results do not take into account several biomarkers, which could have an impact on the prognosis of CC, such as microsatellite instability and mismatch repair status. Future studies on the subject should attempt to address these weaknesses.

DISCUSSION

We presented the results that validate the use of TB to predict postoperative tumor relapse and the OS of patients with stage II and III CC. In our study patients with high-grade TB had significantly higher risk of experiencing tumor relapse and death of any cause in comparison to the patients with low-grade TB. High-grade TB has also been linked to patient population characteristics such as older patients, stage of the disease, and nodal metastases status and lympho-vascular invasion. Furthermore, descriptive statistics in our study showed that patients with lung and liver postoperative relapses had higher percentage of cases with high-grade TB (94.1%). In a cohort study, Smit M. A et al. out of 246 cases found that the high grade TB (≥ 10 buds) group showed a significant worse prognosis compared to the low and intermediate grade group. In their study they analyzed the value of the tumor-stroma rotation (TSR) and TB in stage II and stage III CC patient samples and the high-grade TB group was small with 17 cases (7%). We could speculate that this is comparable with our results, despite the fact that it is a study on a larger group of patients [32].

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We found that the patients with registered high-grade TB demonstrated significantly lower RFS and OS rates than the patients with low-grade TB over the observation period (RFS: 53.8% vs. 98.5%; OS: 65.4% vs. 97.1%, respectively). These results are in line with the study of Basile et al. where by using analysis on scanned H&E tissue sample slides TB has been assessed in three categories: TB1 (0-4 buds: low), TB2 (5-9 buds: intermediate); and TB3 (≥ 10 buds: high). They found considerably worse 3-year DFS in patients with TB2 and TB3 compared with TB1 patients. However, the same findings were found when patients from TB2 and TB3 group were placed together in juxtaposed with the TB1 patients. Studies with similar results have been also reported.

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CONCLUSION

In conclusion, even though the study has been limited due to the relatively small number of analyzed patients, the results revealed a higher number of patients with TB1 than with TB2 and TB3 in our group of patients from the Republic of North Macedonia. The survival analyses showed that the group of TB2 and TB3 CC patients are associated with worse RFS and OS in stage II and III CC patients in opposite to the patients with TB1. Based on this, our results, according to IT-BCC 2016 recommendations, are highly suggestive that TB should be included as a histological biomarker in the pathology report of patients with stage II and stage III CC, because of its prognostic value. Further analyses on larger cohort of patient is required.

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Резиме

ТУМОРСКОТО ОТКАПУВАЊЕ КАКО ПРОГНОСТИЧКИ МАРКЕР КАЈ ПРИМАРНИОТ ДЕБЕЛОЦРЕВЕН КАРЦИНОМ – ИСКУСТВА ОД ЕДИНИЧЕН ЦЕНТАР

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Вовед: Туморското откапување (ТО) се смета за релевантен морфолошки и прогностички фактор кај дебелоцревниот карцином (ДЦК). Целта на оваа студија е да се одреди ТО и да се процени неговата поврзаност со клиничко-патолошките наоди кај пациентите со примарен ДЦК во II и III стадиум, како искуство од единичен центар.

Материјали и методи: Во оваа ретроспективна студија беа опфатени вкупно 120 пациенти со ДЦК, оперирани во периодот од 2018 до 2021 година на Универзитетската клиника за дигестивна хирургија во Скопје, РС Македонија. ТО беше евалуирано на 200x зголемување по должината на инвазивниот туморски фронт на препарати боени со H&E и имунохистохемиски со СКАЕ1/АЕ3. Беа користени две категории: низок степен (ТВ1, 0–4 TVs) и висок степен на ТО, кој вклучува среден степен (ТВ2, 5–9 TVs) и висок степен (ТВ3 $\geq$ 10 TVs) на ТО.

Резултати: Идентификувана е статистички значајна корелација меѓу високиот степен на ТО и возраста ($p = 0,05$) на пациентите, како и значително поголема појава на висок степен ТО кај пациенти во стадиум III со ДЦК. Исто така, пронајдени се статистички значајни корелации во статусот на лимфните јазли ($p < 0,01$), васкуларната инвазија ($p < 0,05$), лимфната инвазија ($p < 0,01$), постоперативниот релапс ($p < 0,01$) и смртта ($p < 0,01$). Постоперативниот релапс и смртта беа значително почести кај пациентите со висок степен на ТО во однос на оние со низок степен на ТО. Пациентите со висок степен на ТО покажаа значително пониски стапки на преживување без релапс и севкупно преживување, од пациентите со низок степен на ТО во текот на периодот на набљудување (RFS: 53,8 % наспроти 98,5 %, $p < 0,001$; OS: 65,4 % наспроти 97,1 %, $p < 0,001$, соодветно). Пациентите со постоперативен релапс во белите дробови и црниот дроб имале поголем процент на случаи со висок степен на ТО (94,1 %).

Заклучок: Нашите резултати сугерираат дека е потребно ТО да биде вклучен како хистолошки биомаркер во патолошките извештаи кај пациенти со ДЦК во стадиум II и III, поради неговата прогностичка вредност.

Клучни зборови: дебелоцревен карцином, туморско откапување, преживување, релапс, прогностички маркер