ORIGINAL PAPER

RJME
Romanian Journal of Morphology & Embryology
http://www.rjme.ro/

Immunohistochemical evaluation of tumor budding in colorectal cancer: an important parameter with prognostic value

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Abstract

We analyzed 82 patients with colorectal cancer (CRC) [75 patients with mucinous adenocarcinoma (ADK) and seven patients with "signet ring cell" ADK] using multi-cytokeratin (CK) AE1/AE3 immunohistochemical assay. In order to determine the mucinous nature of some of the lymph node metastases of the mucinous colorectal ADKs studied, Periodic Acid Schiff–Alcian Blue (PAS–AB) histochemical staining was used. The counting results were systematized in the following ranges: 0 budding areas; between 1–4 budding areas; between 5–9 budding areas; and ≥10 tumor budding (TB) areas. The statistical analysis was performed using the Student's *t*-test. More than half of the cases of mucinous ADK revealed an increased intensity of TB, whereas in the case of "signet ring cell" ADK, an average intensity of this phenomenon. Mucinous ADKs, which were pT3 staged, showed an increased intensity of TB, and those in pT2 stage demonstrated, in the vast majority of cases, the absence of TB. There was a predominance of TB intensity in the absence of vascular-lymphatic invasion. Our study shows the existence of a concordance between tumor progression, the histological type of CRC, vascular-lymphatic invasion and the phenomenon of TB.

Keywords: tumor budding, colorectal cancer, immunohistochemical evaluation, prognostic value.

Introduction

Tumor budding (TB), defined by the existence of isolated or small cell groups (maximum five cells) at the invasive front stroma of carcinoma, is the consequence of the detachment of such cells from the primary tumor (pT), being an early stage of the local invasion and distant metastasis [1]. Recent studies have shown that epithelial–mesenchymal transition-related proteins [zinc finger E-box binding homeobox 1 (ZEB1), TWIST, SNAIL and SLUG] play a minor role in the formation of tumor buds [2].

Retrospective studies on TB in colorectal cancer (CRC) patients have established that this phenomenon is related to the following clinical situations: in the preoperative period, TB on biopsy can indicate neoadjuvant therapy; in patients with malignant colon polyps, the presence of TB on the resection polyps indicates the risk of lymph nodal dissemination and colectomy is required; in patients with stage II colon cancer, the presence of TB is a predictive factor requiring adjuvant therapy [3].

Aim

In the current study, we have proposed to investigate the possible correlations between TB and histological parameters [histological type, tumor progression with pT staging, vascular-lymphatic invasion, lymph nodal and distant metastases].

Patients, Materials and Methods

Patient data

Initially, we studied a number of 805 patients with CRCs, hospitalized and operated in the IInd Department of Surgery, Emergency County Hospital, Craiova, Romania, during 2007–2016. We excluded from this study the patients who received radiotherapy (65 cases), patients with tumor recurrence (positive resection margins – 22 cases), patients with immunohistochemical (IHC) examination not possible (25 cases), patients for whom a postoperative follow-up of more than six months (45 cases) was not possible, and patients with non-mucinous colorectal adeno-

carcinoma (ADK) (570 cases). There were 82 patients in the study. The age of patients remaining in the study was between 31 and 94 years old, with predominance of the male gender (56%).

The study was approved by the Institutional Ethics Committee, and informal consent was obtained from all participating patients.

Histopathological examination

The study material consisted of colon resection specimens (right hemicolectomy, left hemicolectomy, total/subtotal colectomy, segmental colectomy, and recto-sigmoid resection). Seventy-five cases of mucinous (colloid) ADK (three of which were primarily multiple malignancies at the colorectal level), and seven cases of "signet ring cell" ADK were analyzed. Of the three cases of multiple primary malignancies at the colorectal level, two cases were tumor synchronisms, localized within the same intestinal segment (right colon) and consisted of simultaneous diagnosis of mucinous ADK and other non-mucinous ADKs. The third case of multiple primary malignancies was a metachronous tumor that consisted in the development of a mucinous colon carcinoma in a patient diagnosed one year ago with gastrointestinal stromal tumor (GIST) of the small intestine. Also, one patient with recto-sigmoid collision tumor (association within the same tumor, mucinous (colloid) ADK and neuroendocrine tumor) was also present within the mucinous (colloid) ADKs.

Extracted surgical tissue fragments were fixed within three days in 10% neutral buffered formalin solution. Then, 1 cm^2 fragments were included in paraffin and 5 μm sections were stained with Hematoxylin–Eosin (HE) to establish histopathological (HP) diagnosis and special staining techniques [Periodic Acid Schiff (PAS)–Alcian Blue (AB) to highlight mucin (MUC)].

Immunohistochemistry

We used IHC labeling with the antibody system called Bond™ ready-to-use primary antibody multi-cytokeratin (CK) AE1/AE3 (Catalog No. PA0909) to visualize the phenomenon of TB. Anti-CK AE1/AE3 antibody is intended to be used for the quantitative identification by light microscopy of human CKs 56.5, 50.5, 48 and 40 kD of acidic subfamily, and 65 to 67, 64, 59, 58 and 52 kD of basic subfamily, in formalin-fixed paraffin-embedded tissue by IHC staining using the automated Bond™ system. AE1 clone recognizes the 56.5, 50.5, 48, and 40 kD human CKs of the acidic subfamily. AE3 clone recognizes the 65 to 67, 64, 59, 58 and 52 kD human CKs of the basic subfamily.

For each case in the study group, representative fragments for the presence and study of lymph nodes in adipose tissue adjacent to colorectal tumors were collected and processed (by fixation in 10% neutral buffered formalin solution) Fragments were processed by inclusion to paraffin, then cut to 2–3 μm thick and usually stained by HE. In order to determine the mucinous nature of some of the lymph node metastasis of the mucinous colorectal ADKs studied, PAS–AB histochemical staining was used. For colonic collision tumor-specific lymph node metastases (of which one of the mucosal type) included in the study group, the IHC marker was used with the EnVision™ G/2 Doublestain System, rabbit/mouse [3,3'-Diaminobenzidine (DAB)+/Permanent Red]. The IHC antibody panel used consisted of: anti-CK AE1/AE3 (clone AE1/AE3, IgG1 kappa isotype), anti-MUC2 (clone NCL, IgG1 kappa isotype), anti-chromogranin A (clone LK2H10, IgG1 rabbit polyclonal). Subsequently, visualization was performed using Dako Labeled Streptavidin–Biotin (LSAB)+/Horseradish peroxidase (HRP) and Dako EnVision+/HRP and DAB chromogen.

Evaluation

Morphometric analysis was used to quantify the density of TB, in order to focus on the local and prognostic invasive capacity of the CRCs that comprised the studied group. For this purpose, the presence of tumor cells isolated or arranged in small groups of up to five cells, at the level of the tumor invasion front, was identified by multi-CK AE1/AE3 IHC staining (cytoplasmic brown marker). The method used consisted in counting the budding areas from a microscopic field (200×), or at the tumor interface with normal colorectal mucosa, or at the depth of the stroma (in the tumor area of maximal expression of this phenomenon). The counting results were systematized in the following ranges: 0 budding areas; between 1–4 budding areas; between 5–9 budding areas; and ≥10 TB areas (algorithm adapted after Ohtsuki *et al.*) [4]. Acquisition of the images was done using the Leica microscope (Japan). The images were acquired at the 20× and 40× lenses, for each image checking the signal and then the validity of the reception. Image processing was done using Adobe Photoshop 7.0.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software package that implement the specific statistical analysis algorithms needed for the study. Clinical-pathological, HP, IHC and morphometric data were recorded in an electronic database that used the Microsoft Access 2010 Platform and formed the basis of the batch of patients that were statistically studied. The test used was the Student's *t*-test. The correlation relationships between the measured parameters were highlighted by calculating some correlation coefficients (p), following the evaluation of the mutual influence between two factors. For this purpose, we used the following interpretation of the p values, values provided directly by the program with which the statistical data processing was performed: $p<0.05$ (the difference between the two parameters is significant); $p<0.01$ (the difference between the two parameters is highly significant); $p<0.001$ (the difference between the two parameters is very highly significant); $p>0.05$ (the difference between the two parameters is not significant). In the statistical analysis, the obtained results suggested that TB, evidenced by multi-CK AE1/AE3 immunoexpression, was associated with HP parameters, such as HP type ($p=0.00196$), higher pT stage ($p=0.006186$) and vascular-lymphatic invasion ($p=0.01556$), and not with lymph node and distant metastases (Table 1).

Table 1 – ***p-values using Student's t-test***

Parameter	*p*-value (>0.05)
Histopathological type	0.00196
Tumor progression	0.006186
Vascular-lymphatic invasion	0.01556
Lymph node metastases	0.4236
Distant metastases	0.1038

Results

Correlations TB–histological parameters

TB–histopathological type

The analysis of TB by using the multi-CK AE1/AE3 immunoassay for the 82 cases of mucin-secreted CRCs revealed the existence of this mechanism in 52 cases (63.41% of the total study group). Multi-CK AE1/AE3 IHC results for the TB expression are shown in Figure 1. Multi-CK AE1/AE3 immunoassay of the budding areas was considered positive by brown staining at the invasion front of isolated tumor epithelial cells or in the groups consisting of up to five tumor cells. An irregular appearance of isolated tumor epithelial cells constituting the budding area has been noted, in some cases these cells have pseudopodium-like cytoplasmic protrusions or glandular luminescence sketches made by cell groups in the budding area. Multi-CK AE1/AE3 positive immunoassay had a different statistical distribution, depending on the degree of tumor progression, with significantly different values in terms of the type of carcinoma.

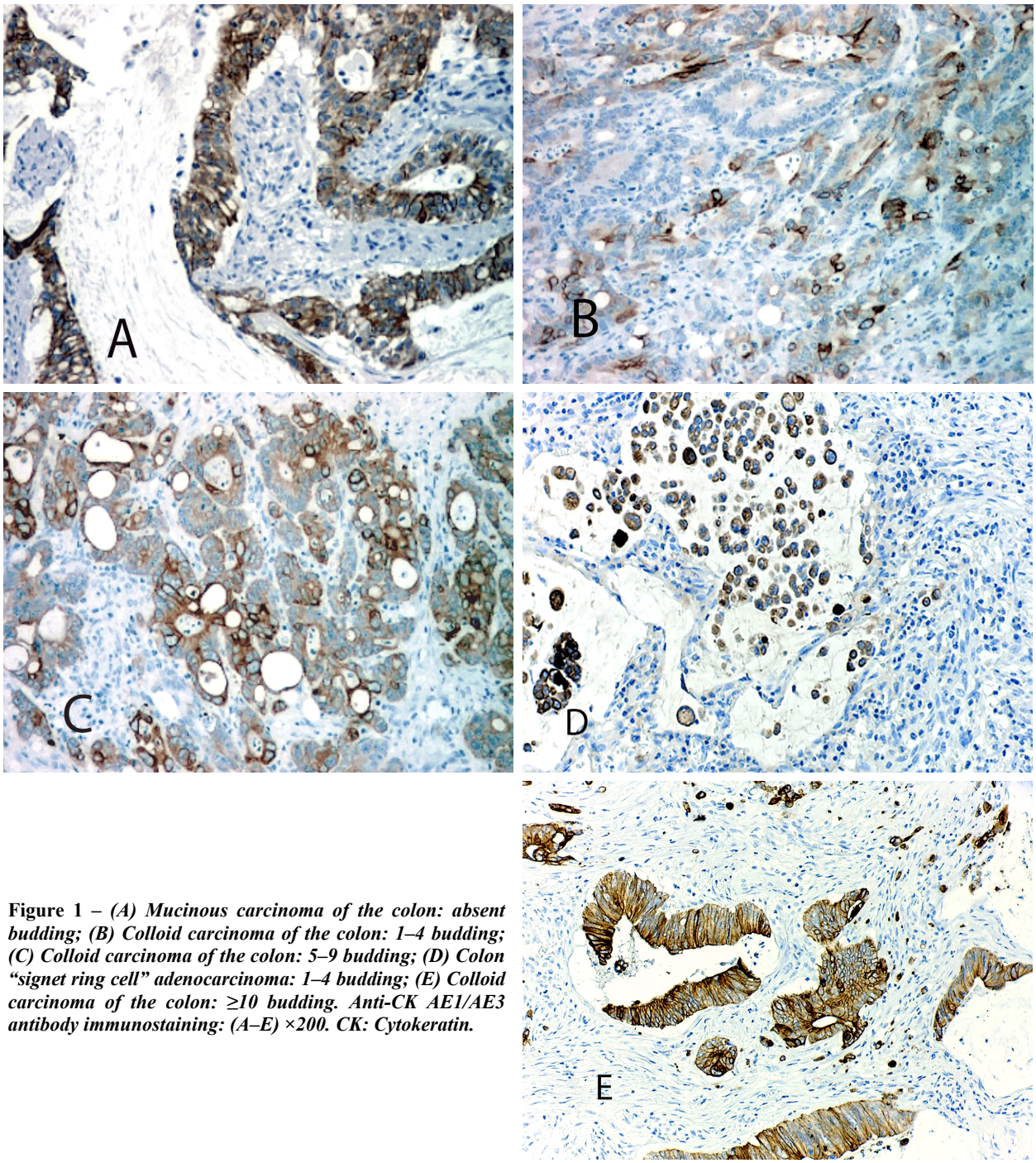

Figure 1 – ***(A) Mucinous carcinoma of the colon: absent budding; (B) Colloid carcinoma of the colon: 1–4 budding; (C) Colloid carcinoma of the colon: 5–9 budding; (D) Colon "signet ring cell" adenocarcinoma: 1–4 budding; (E) Colloid carcinoma of the colon: ≥10 budding. Anti-CK AE1/AE3 antibody immunostaining: (A–E) ×200. CK: Cytokeratin.***

Of the total of 52 mucinous CRCs, in which TB was identified, it was expressed with increased intensity (over 10 budding areas/microscopic field) in a significant number of cases (23/52 cases, 28.04%). Ten (12.19%) cases were in the range of 1–4 budding areas/microscopic field, and 19 (23.17%) cases were in the range of 5–9 budding areas/ microscopic field (Figures 2 and 3).

Of the 75 colloid carcinoids studied, 23 (30.66%) cases revealed increased intensity of budding (more than 10 budding areas/microscopic field) (Figure 1E), 18 cases with average intensity (5–9 budding areas/microscopic field, 24%) (Figure 1C), there were 28 (37.33%) cases in which this phenomenon was not evidenced (Figure 1A) and six cases that had the phenomenon of TB manifested in a low form (1–4 budding areas/microscopic field) (Figure 1B). "Signet ring cell" ADK expressed low-intensity budding (1/7 cases, 14.28%) and in appreciable numbers (4/7 cases) of medium intensity (1–4 budding areas/microscopic field) (Figure 1D).

There was no histological case of the "signet ring cell" ADK to intensely express the phenomenon of TB (Figure 3).

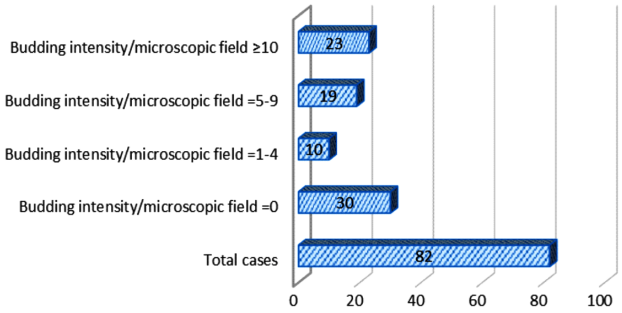

Figure 2 – ***Analysis of tumor budding by using multi-CK AE1/AE3 immunoassay. CK: Cytokeratin.***

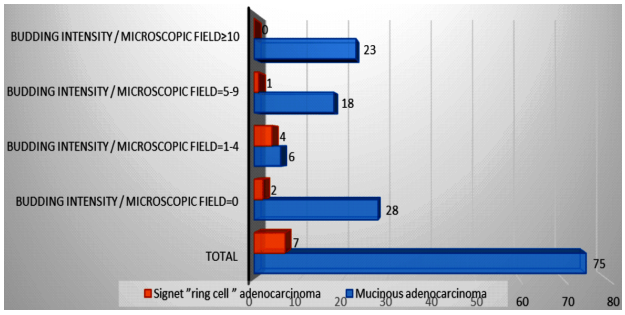

Figure 3 – ***Analysis of the intensity of tumor budding according to the histopathological type.***

TB–tumor progression

In relation to tumor progression, mucinous cancers in the studied pT2 stage demonstrated in the vast majority (7/8 cases, 87.5%) the absence of TB, there being only one case where the TB was highlighted with low intensity (1–4 budding areas/microscopic field) (Figure 4). The mucinous carcinomas in the study group, which were pT3 staged, showed significant (≥10 budding areas/microscopic field) phenomena in significant numbers (21/65 cases, 32.3%). Cancers at the same stage and in the budding intensity range (5–9 budding areas) were significant (18/65 cases, 27.69%). For the pT4 stage, the study demonstrated the existence of only two (22.22%) cases with budding phenomenon of medium intensity and in one case, the phenomenon of high intensity budding (≥10 budding areas/microscopic field) (Figure 4).

TB–vascular-lymphatic invasion

In relation to vascular-lymphatic invasion, the TB phenomenon was observed with maximum intensity (5–9 budding areas) in the absence of vascular-lymphatic invasion in 28 out of 73 cases (Figure 5).

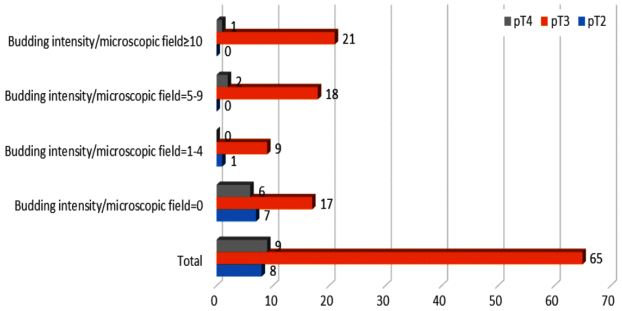

Figure 4 – ***Analysis of the intensity of tumor budding according to tumor progression.***

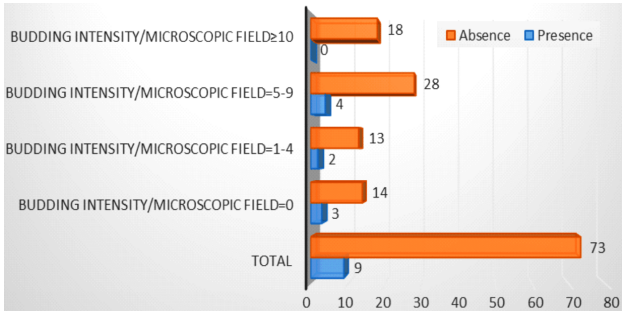

Figure 5 – ***Analysis of the intensity of tumor budding according to vascular-lymphatic invasion.***

TB–lymph node metastases

The metastatic potential of CRCs reflected by the presence of distant and lymph node metastases by multi-CK AE1/AE3 immunoexpression revealed the existence of a significant number of such cases with high- and medium-intensity budding phenomenon: 10/29 (34.4%) cases intensive budding and 9/29 cases with medium intensity budding (1–4 areas of budding), in the case of CRCs with lymph node metastases, and in the case of distant metastases, 4/8 (50%) cases of intensive budding (≥10 budding areas/microscopic field) and 2/8 (25%) cases of medium-intensity budding (1–4 budding areas/ microscopic field) (Figure 6).

TB–distant metastases

The distant metastases of the mucinous carcinomas in the studied group were manifested by the presence of the budding phenomenon, with 50% of cases expressing the maximum intensity of the TB. The majority (47.9%) of cancers studied with lymph node metastases showed moderate budding (5–9 budding areas/microscopic field), with a significant number of cases (34.4%) with intense budding (≥10 budding areas/microscopic field) (Figure 7).

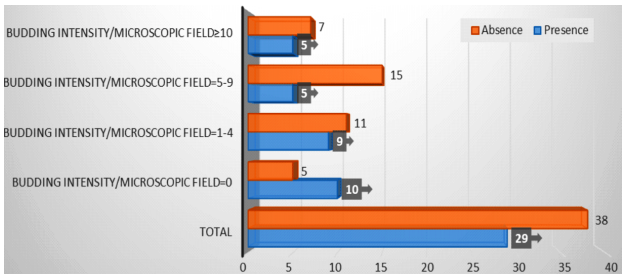

Figure 6 – *Analysis of the intensity of tumor budding according to lymph node metastases.*

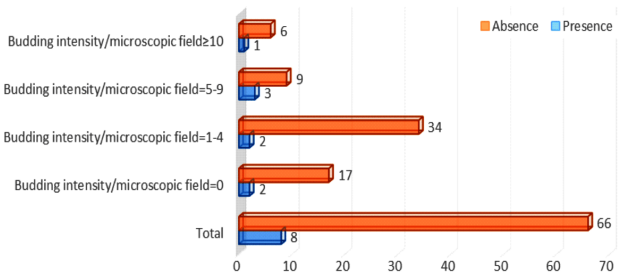

Figure 7 – *Analysis of the intensity of tumor budding according to distant metastases.*

Discussions

The *International Tumor Budding Consensus Conference* (ITBCC) in 2016 established that TB is an independent predictor for lymph node metastases in pT1 CRC patients and is an independent survival predictor in patients with stage II CRC. It has also been established that TB is linked to the potential for lymphangial metastasis of patients with CRC, must be introduced into the therapeutic protocols and staging systems of the patients with CRC, and the evaluation should be done on HE-stained sections in a microscopic field measuring 0.785 mm^2 [5].

Okuyama *et al.* indicated the existence of 58% of the TB phenomenon in the study of mucinous CRCs, the reported value being similar to the result of our study [6]. Also, Ohtsuki *et al.* identifies in a CRC study a value of 34 cancers of ≥10 areas/microscopic field, a percentage value similar to our study [4].

The studies performed show a very broad expression of the intensity of the TB phenomenon depending on the histological variety of the mucin secreting CRCs and there are studies [7] in which the TB was absent in the "signet ring cell" ADK, while other studies [8] highlighted an intense TB of both histological varieties.

Numerous similar specialty studies [4, 6] state as a result of the research that there is a concordance between the TB phenomenon and the stage of tumor progression of CRCs, which is the conclusion of our study as well.

The lymphovascular and perineural invasion [9–16], as well as TB, are predictive factors with risk of lymph node metastasis [4, 17]. Numerous studies, investigating the correlation of prognostic factors with the TB phenomenon, have reported a close concordance between vascular-lymphatic invasion (especially lymphatic) and TB [18], and between peritoneal dissemination and mucinous budding-positive CRCs [6].

The study by Cappellesso *et al.* found that there was a strong correlation between TB and the risk of lymph node metastasis in patients with CRC pT1, indicating that patients with a history of endoscopic resection of colon polyps should establish the existence of TB (in the case of malignant colon polyps with TB present, colectomy should be considered) [19].

The paper of Mehta *et al.*, on a study group of 60 patients, using multi-CK AE1/AE3 immunoassay, revealed that there is a statistically significant correlation between the TB and lymph node invasion, on the one hand, and between TB and *American Joint Committee on Cancer* (AJCC) staging of CRC, on the other hand, which highlights the fact that TB can be considered a promising predictor of lymphoganglionar invasion in patients with CRC [20].

Conclusions

There is a concordance between the stage of tumor progression in patients with CRC and the phenomenon of TB. The phenomenon of TB was expressed with high intensity in patients with CRC in pT3 stage. Our study shows the existence of a concordance between the histological type of CRC, vascular-lymphatic invasion and the phenomenon of TB.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

The authors would like to thank the reviewers for their helpful comments and acknowledge the support of the research grant No. 26/2014, code PN-II-PT-PCCA-2013-4-1153, entitled IMEDIATREAT – Intelligent Medical Information System for the Diagnosis and Monitoring of the Treatment of Patients with Colorectal Neoplasm – financed by the Romanian Ministry of National Education (MEN) – Research and the Executive Agency for Higher Education Research Development and Innovation Funding (UEFISCDI).

References

[1] Marzouk O, Schofield J. Review of histopathological and molecular prognostic features in colorectal cancer. Cancers (Basel), 2011, 3(2):2767–2810.
[2] Yamada N, Sugai T, Eizuka M, Tsuchida K, Sugimoto R, Mue Y, Suzuki M, Osakabe M, Uesugi N, Ishida K, Otsuka K, Matsumoto T. Tumor budding at the invasive front of colorectal cancer may not be associated with the epithelial–mesenchymal transition. Hum Pathol, 2017, 60:151–159.
[3] Koelzer VH, Zlobec I, Lugli A. Tumor budding in colorectal cancer – ready for diagnostic practice? Hum Pathol, 2016, 47(1):4–19.
[4] Ohtsuki K, Koyama F, Tamura T, Enomoto Y, Fujii H, Mukogawa T, Nakagawa T, Uchimoto K, Nakamura S, Nonomura A, Nakajima Y. Prognostic value of immunohistochemical analysis of tumor budding in colorectal carcinoma. Anticancer Res, 2008, 28(3B):1831–1836.
[5] Lugli A, Kirsch R, Ajioka Y, Bosman F, Cathomas G, Dawson H, El Zimaity H, Fléjou JF, Hansen TP, Hartmann A, Kakar S, Langner C, Nagtegaal I, Puppa G, Riddell R, Ristimäki A, Sheahan K, Smyrk T, Sugihara K, Terris B, Ueno H, Vieth M, Zlobec I, Quirke P. Recommendations for reporting tumor budding in colorectal cancer based on the International Tumor Budding Consensus Conference (ITBCC) 2016. Mod Pathol, 2017, 30(9):1299–1311.

[6] Okuyama T, Oya M, Yamaguchi M. Budding (sprouting) as a useful prognostic marker in colorectal mucinous carcinoma. Jpn J Clin Oncol, 2002, 32(10):412–416.
[7] Kang SH, Chung WS, Hyun CL, Moon HS, Lee ES, Kim SH, Sung JK, Lee BS, Jeong HY. A rare case of a signet ring cell carcinoma of the colon mimicking a juvenile polyp. Gut Liver, 2012, 6(1):129–131.
[8] Pai RK, Jayachandran P, Koong AC, Chang DT, Kwok S, Ma L, Arber DA, Balise RR, Tubbs RR, Shadrach B, Pai RK. *BRAF*-mutated, microsatellite-stable adenocarcinoma of the proximal colon: an aggressive adenocarcinoma with poor survival, mucinous differentiation, and adverse morphologic features. Am J Surg Pathol, 2012, 36(5):744–752.
[9] Meşină C, Vasile I, Ciobanu D, Calotă F, Gruia CL, Streba L, Mogoantă SŞ, Pârvănescu H, Georgescu CV, Tarniţă DN. Collision tumor of recto-sigmoidian junction – case presentation. Rom J Morphol Embryol, 2014, 55(2 Suppl):643–647.
[10] Ciobanu D, Meşină C, Streba L, Gruia CL, Diţescu D, Şarlă CG, Enescu A, Petrescu F. The role of immunohistochemistry in diagnosis a synchronous colon tumor. Rom J Morphol Embryol, 2014, 55(3 Suppl):1203–1213.
[11] Meşină C, Mogoantă SŞ, Cristian DA, Dumitrescu TV, Drăgoescu PO, Meşină-Botoran MI, Ciurea ME, Ghiluşi MC, Ciobanu D. Retroperitoneal ancient schwannoma – case presentation. Rom J Morphol Embryol, 2015, 56(4):1517–1522.
[12] Meşină C, Vasile I, Vîlcea ID, Paşalega M, Pârvănescu H, Calotă F, Georgescu CV, Ghiluşi M, Dumitrescu T, Mirea C, Mogoantă S, Moraru E. [Soft tissue sarcoma – problems of diagnosis and treatment]. Chirurgia (Bucur), 2010, 105(2): 257–266.
[13] Meşină C, Vasile I, Vîlcea ID, Vere CC, Georgescu CV, Ghiluşi M, Paşalega M, Pârvănescu H, Calotă F, Mogoantă SŞ. Axillary and perianal leiomyosarcoma: report two cases. Rom J Morphol Embryol, 2010, 51(2):379–385.
[14] Mesina C, Dumitrescu TV, Calota F, Ciobanu D. Letter to the Editor. An uncommon abdominal fistula: colonic diverticular disease complicated with colocutaneous fistula. Turk J Gastroenterol, 2018, 29(2):247–248.
[15] Meşină C, Dumitrescu TV, Mogoantă SŞ, Ciorbagiu MC, Cristian DA, Meşină-Botoran MI, Gruia CL, Foarfă MC, Meşină M, Ciurea ME, Ciobanu D. An unusual cause of acute surgical abdomen: benign multicystic peritoneal mesothelioma associated with adenomatous tumor. Rom J Morphol Embryol, 2018, 59(3):971–976.
[16] Mesina C, Stoean LC, Stoean R, Sandita VA, Gruia CL, Foarfa MC, Rotaru LT, Ciobanu AE, Mesina M, Calborean V, Gheorman V, Ciobanu D. Immunohistochemical expression of CD8, CDX2, p53, D2-40 and Ki67 in colorectal adenocarcinoma, conventional and malignant colo-rectal polyps. Rev Chim (Bucharest), 2018, 69(2):419–428.
[17] Huh JW, Kim HR, Kim YJ. Lymphovascular or perineural invasion may predict lymph node metastasis in patients with T1 and T2 colorectal cancer. J Gastrointest Surg, 2010, 14(7): 1074–1080.
[18] Morodomi T, Isomoto H, Shirouzu K, Kakegawa K, Irie K, Morimatsu M. An index for estimating the probability of lymph node metastasis in rectal cancers. Lymph node metastasis and the histopathology of actively invasive regions of cancer. Cancer, 1989, 63(3):539–543.
[19] Cappellesso R, Luchini C, Veronese N, Lo Mele M, Rosa-Rizzotto E, Guido E, De Lazzari F, Pilati P, Farinati F, Realdon S, Solmi M, Fassan M, Rugge M. Tumor budding as a risk factor for nodal metastasis in pT1 colorectal cancers: a meta-analysis. Hum Pathol, 2017, 65:62–70.
[20] Mehta A, Goswami M, Sinha R. Histopathological significance and prognostic impact of tumor budding in colorectal cancer. Ann Clin Lab Sci, 2017, 47(2):129–135.

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Received: April 6, 2019

Accepted: December 21, 2019