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Correlation between Tumor Budding, MVD in Colorectal Carcinoma

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Abstract: Colorectal cancer is one of the leading causes of cancer death in both developed and developing nations. Therefore, identification of clinicopathological prognostic factors associated with tumor growth, invasion and metastasis has been critical to develop potential therapeutic interventions. Tumor budding is a high-grade undifferentiated component of a tumor at the leading invasive edge as small clusters (<5) or single infiltrating tumor cells. The current study included fifty cases of colorectal carcinomas studied histologically for tumor budding and immunohistochemically for MVD by CD34. Tumor budding was observed in 62% of cases and it showed a statistical significant correlation with advanced Dukes' stage, positive lymph node metastasis and increasing stage of lymph node metastasis. MVD showed significant correlation only with the sex of patient, but no correlation with tumor budding. In conclusion, this study suggests that tumor budding might play a role in the ability of colon cancer cells to invade and metastasize and thus may be used as a prognostic marker to predict poor outcome of patients and a scoring system for pathological reporting of tumor budding may be beneficial. It will be necessary to carry out similar studies on a larger sample size to predict the possible prognostic significance of MVD.

Key words: Colorectal carcinoma · Budding · MVD

INTRODUCTION

Colorectal cancer (CRC) is the fourth leading cause of cancer-related mortality in the world [1]. In Egypt, the Cancer Pathology Registry of National Cancer Institute showed that during 2003-2004, colorectal cancer was the commonest among digestive tract malignancies (15.78%) and the fifth among all cancers (4.34%) [2]. Early diagnosis and treatment of CRC, knowledge of its clinicopathological prognostic factors and response to adjuvant therapy have contributed to better outcome of affected patients. Therefore, identification of new molecular markers associated with tumor growth, invasion and metastasis is critical to develop potential therapeutic intervention [3]. In a study done by Gabbert *et al.* [4] observed that at the invasive borders of differentiated adenocarcinomas, there were small strands and cords of tumor cells projecting from the neoplastic glands and at the deepest border discontinuous small aggregates or single tumor cells were found. Ultrastructurally, these tumors cells did not elaborate junctional complexes, had incomplete desmosomes and a basement membrane was absent or rudimentary. Tumor budding represents a high-grade undifferentiated component of a tumor at the leading invasive edge as small clusters (<5) or single infiltrating tumor cells [5]. According to Ueno *et al.* [6], budding foci were defined as clusters composed of fewer than five undifferentiated cancer cells. Tumor budding and tumor cell migration at the invasive margin by pseudopodia has been proposed by Shinto *et al.* [7]. Tumor budding may indicate high risk of recurrence after surgery and provides grading by the worst pattern. This correlates with loss of adhesion molecules [8] and increased metalloproteinase expression [9]. Tumor budding implies a higher risk for lymph node metastasis of early stage CRC [10]. The CD34 family of cell-surface proteins comprises the hematopoietic progenitor cell antigen [11]. CD34 is used as a marker of vascular endothelial cells, in the identification and isolation of hematopoietic stem cells (HSCs) and progenitors for bone-marrow transplantation and to help identify other tissue-specific stem cells [12]. Tumors cannot enlarge beyond 1 to 2mm in maximal dimension unless they are well vascularized. Angiogenesis is mandatory for continued tumor growth and for metastasis [13]. Microvascular density (MVD) correlates with stage and grade of tumor and CD34 expression in CRC correlates with recurrence and metastasis [14].

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The aim of the current study is to evaluate tumor budding, detection of MVD by CD34 in 50 cases of colorectal carcinoma, then to correlate between tumor budding, MVD and other clinical and pathological features to determine their possible role in tumor progression and their prognostic value.

MATERIALS AND METHODS

This work included 50 cases of colorectal carcinoma obtained through collection of archived paraffin blocks. All cases obtained were through colectomy specimens. Data obtained from pathology sheet are: age and sex of patient, site of colorectal carcinoma, size of the tumor, growth appearance, presence of distant metastasis and surgical margins whether positive or negative. Serial sections of 4 microns thick were prepared from each block, one of them was mounted on glass slide and stained by Hematoxylin and Eosin (H&E) for histological evaluation and another two were mounted on charged slides for immunohistochemical staining.

Histological Examination: Histopathological examination of H&E stained slides was performed for: histological tumor type and histological grade. Staging was done according to Modified Duke's stage. The depth of tumor invasion, lymph node metastasis and distant metastasis were also assessed according to TNM staging. The budding number was determined via counts in one microscopic field × 200 in an area of maximal budding. The degree of budding was classified as negative or positive corresponding to 0-4 and =5 budding foci in one field, respectively, using HE-stained specimens [15].

Immunohistochemical Staining: The sections were deparaffinized in xylene, then were hydrated through a series of graded alcohols (95%-70%), distilled water and phosphate buffered saline (ph 7.5). The slides were then immersed in 10mm citrate buffer (ph 6) and were twice pretreated by microwaving oven 800w for 4 then 8 minutes. After a 25 minute cooling period, the endogenous peroxidase activity was inhibited by incubation in 3% hydrogen peroxide for 5 minutes. Antigen retrieval was done by immersing the slides in 10mm citrate buffer (pH 6) for 10-20 minutes at 100°C in a microwave followed by cooling at room temperature for 20 minutes. The tissues were blocked with protein blocking reagent for 30 minutes to reduce nonspecific staining. After washing with Tris-buffered saline, the sections were incubated with the primary antibody over night at room temperature. The primary antibody for CD34, mouse monoclonal antibody, anti-CD34 Clone (QBend10) (Genamid Biotechnologies Company).The sections were washed in Tris-buffer and incubated with avidin-biotin-peroxidase system (DAKO) for 30 minutes. The excess reagent was tapped off and the slides were washed with PBS and dried. Peroxidase reaction was detected by addition of diamonobenzidinetetrahydrochloride. Two or three drops of streptavidin enzyme label were placed on each slide for 30 minutes at room temperature. The excess reagent was tapped off and the slides were washed with PBS and dried. All slides were rinsed well in tap water for 5 minutes then slightly counterstained with Hematoxylin for 1-2 minutes and dehydrated in ascending alcohol.

Evaluation of CD34 Expression (MVD): Microvessel density (MVD) was assisted by scanning the entire tumor section at low magnification (×100) to identify the most intense areas of neovascularization ("hotspots"). After five hotspots areas with the highest number of capillaries and small venules were identified, microvessels were counted at high power magnification (× 400) and the average of count in five fields was calculated. MVD was quoted as a continuous variable. Cases below the average MVD considered to be cases with low MVD, while cases more than or equal to average considered to be cases with high MVD (16).

Statistical Methods: The data collected were analyzed using SPSS version 16.0 and evaluation of relationship of tumor budding, â-catenin expression and MVD in colorectal carcinoma to each other and to other clinicopathologic features was done. Determining the probability factor (p value) assessed the significance of results. When P value levels were found to be less than 0.05 the results were considered to be statistically significant.

RESULTS

This retrospective study was conducted on fifty CRC cases: 23 were males and 27 were females, with a female to male ratio of 1.17:1. Their ages ranged from23to86years with a mean of 49.32±12.84 years. Forty seven out of fifty cases studied (94%) were □40 years and 3(6%) were ≤40 years. The commonest location of tumors was the colon (ascending, transverse and descending), representing (56%) 28 cases, while 22 cases (44%) were located in the rectum. Twenty eight cases (56%) showed tumor size □5 cm and 22 cases (44%) were ≤ 5cm.Twenty one cases (42%) manifested as ulcerating tumor, nineteen cases

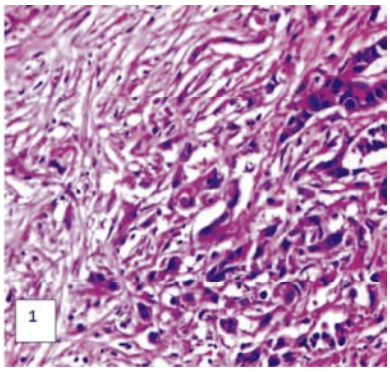

Fig. 1: Moderately differentiated colonic adenocarcinoma showed tumor budding at the invasive front of the tumor (H&E x 400).

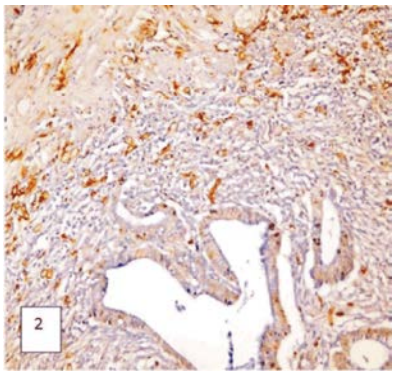

Fig. 2: Moderately differentiated adenocarcinoma associated with high MVD (IPS x 200).

Table 1: Correlations between tumor budding and clinicopathological data of the studied CRC cases.

		Tumor Budding			
Clinicopathological		Positive	Negative	Total	P-value
Age	≤40	3 (100%)	0 (0%)	3 (100%)	0.162
	>40	28 (60%)	19 (40%)	47 (100%)	
Sex	Male	14 (60.9%)	9 (39.1%)	23 (100%)	0.879
	Female	17 (63%)	10 (37%)	27 (100%)	
Tumor site	Rt colon	10(52.6%)	9(47.4%)	19 (100%)	0.410
	Lt colon	1(100%)	0(0%)	1 (100%)	
	Transverse colon	4(50%)	4(50%)	8 (100%)	
	Rectosigmoid	16(72.7%)	6(27.3%)	22 (100%)	
Tumor size	≤5	12(54.5%)	10(45.5%)	22(100%)	0.336
	>5	19(67.9%)	9(32.1%)	28(100%)	
Gross appearance	Ulcer	14(66.7%)	7(33.3%)	21(100%)	0.665
	Infiltrating	12(63.2%)	7(36.8%)	19(100%)	
	Fungating	5(50%)	5(50%)	10(100%)	
Microscopic pictures	Adenocarcinoma	23(57.5%)	17(52.5%)	40(100%)	0.190
	Mucoid carcinoma	8(80%)	2(20%)	10(100%)	
Tumor grade	Grade I	0(0%)	1(100%)	1(100%)	0.325
	Grade II	19(67.9%)	9(32.1%)	28(100%)	
	Grade III	12(57.1%)	9(42.9%)	21(100%)	
Stage	Duke B	6(31.6%)	13(68.4%)	19(100%)	0.002
	Duke C	23(79.3%)	6(20.7%)	29(100%)	
	Duke D	2(100%)	0(0%)	2(100%)	
T stage	T2	0(0%)	2(100%)	2(100%)	0.078
	T3	28(62.2%)	17(37.8%)	45(100%)	
	T4	3(100%)	0(0%)	3(100%)	
Lymph node stage	N0	6(31.6%)	13(68.4%)	19(100%)	0.002
	N1	10(71.4%)	4(28.6%)	14(100%)	
	N2	15(88.2%)	2(11.8%)	17(100%)	
Lymph Node Metastasis	Positive	25(80.6%)	6(19.4%)	31(100%)	0.001
	Negative	6(31.6%)	13(68.4%)	19(100%)	

Table 2: Correlations between MVD and clinicopathological data of the studied CRC cases

		MVD			
Clinico Pathological		□Average	□ Average	Total	P-value
Age	≤40	1(33.3%)	2(66.7%)	3(100%)	0.291
	□40	30(63.8%)	17(36.2%)	47(100%)	
Sex	Male	18(78.3%)	5(21.7%)	23(100%)	0.029
	Female	13(48.1%)	14(51.9%)	27(100%)	
Tumor site	Rt colon	11(57.9%)	8(42.1%)	19(100%)	0.547
	Lt colon	0(0%)	1(100%)	1(100%)	
	Transverse colon	5(62.5%)	3(37.5%)	8(100%)	
	Rectosigmoid	15(68.2%)	7(31.8%)	22(100%)	
Tumor size	≤5	13(59.1%)	9(40.9%)	22(100%)	0.707
	□5	18(64.3%)	10(35.7%)	28(100%)	
Gross appearance	Ulcer	14(66.7%)	7(33.3%)	21(100%)	0.665
	Infiltrating	12(63.2%)	7(36.8%)	19(100%)	
	Fungating	5(50%)	5(50%)	10(100%)	
Microscopic pictures	Adenocarcinoma	27(67.5%)	13(32.5%)	40(100%)	0.109
	Mucoid carcinoma	4(40%)	6(60%)	10(100%)	
Tumor grad	Grade I	0(0%)	1(100%)	1(100%)	0.173
	Grade II	20(71.4%)	8(28.6%)	28(100%)	
	Grade III	11(52.4%)	10(47.6%)	21(100%)	
Stage	Duke B	13(68.4%)	6(31.6%)	19(100%)	0.743
	Duke C	17(58.6%)	12(41.4%)	29(100%)	
	Duke D	1(50%)	1(50%)	2(100%)	
T stage	T2	1(50%)	1(50%)	2(100%)	0.927
	T3	28(62.2%)	17(37.8%)	45(100%)	
	T4	2(66.7%)	1(33.3%)	3(100%)	
Lymph node stage	N0	13(68.4%)	6(31.6%)	19(100%)	0.291
	N1	10(71.4%)	4(28.6%)	14(100%)	
	N2	8(47.1%)	9(52.9%)	17(100%)	
Lymph node Metastasis	Positive	18(58.1%)	13(41.9%)	31(100%)	0.464
	Negative	13(68.4%)	6(31.6%)	19(100%)	

(38%) were infiltrating and ten cases (20%) were fungating. All the studied cases showed negative surgical resection margins. Forty cases (80%) were classified histologically as conventional adenocarcinomas, while 10 cases (20%) were mucoid adenocarcinoma. As regards to the degree of differentiation, 28 (56%) of cases were moderately differentiated (grade II), 21 (42%) were poorly differentiated (grade III) and 1 (2%) were well differentiated (grade I). In the studied cases, carcinomas with modified Duke's stage C were the most commonly encountered tumors; 29 cases (58%) followed by Duke's stage B; 19 cases (38%) then Duke's stage D; 2 cases (4%). Forty five (90%) of cases were stage T3, 3(6%) were T4 and 2 (4%) were T2. Most of the studied cases revealed lymph nodes metastatic deposits (62%). Thirty one cases (62%) had tumor buds (Fig. 1), while 19 cases (38%) were negative for tumor budding. The average MVD in our study was 16.16/5 HPFs. Thirty one cases (62%) showed low MVD (less than average MVD) while 19 cases (38%) showed high MVD (Fig. 2). Statistical analysis revealed statistically significant positive correlation between tumor budding and advanced Duck' stage, positive lymph node metastasis and increasing stage of lymph node metastasis, while with the other studied parameters, no significant correlations were detected (Table 1). The microvascular density showed no significant difference with the different clinicopathological variables except for the sex of patient, as females cases showed higher MVD than male cases (p= 0.029) (Table 2). There was no statistically significant relationship between tumor budding and MVD (p=0.895).

DISCUSSION

The current study aimed to evaluate tumor budding, MVD by CD34 in colorectal carcinoma and to correlate between them and other clinical and pathological features to determine their possible role in tumor progression and their prognostic value. In the present study, 54% cases were females and 46% were males, with a female to male ratio 1.17:1. This is similar to what was reported by Cressey *et al.*[17], where females represented higher incidence; 63% of cases and different from that reported by Messerini *et al.*[18] where females represented the lower incidence; 44%. This difference may be attributed to sample size, sample selection methods or geographic variability. Ages of patients ranged from 23 to 86years with a mean of 49.32±12.84 years. Moreover, (94%) were □40years. These figures are close to those reported in western studies by Office for National Statistics, 2011 [19], where 86% of cases arising in people 60 years or older and those reported by researchers from Tanta Cancer Center, Gharbiah Governorate, Egypt; Zeeneldin *et al.* [20] where age range was from 21to 81 years with 85% of the patients ≤ 65 years. Our figures represent older age groups than those reported by researchers on Egyptian patients with colorectal cancer; Soliman *et al.* [21] stated that majority of cases were ≤40 years and Ibrahim *et al.* [22] reported that 48.7% of patients were younger than 50 years. This difference may be attributed to sample size, sample selection methods and wide geographic areas covering different places in Egypt. The majority of tumors in the present study were located in the colon representing 56% of cases (38% of cases in the right colon, 16% in the transverse colon and 2% in the left colon). The rectum came in the second place representing 44%. This is in agreement with those obtained by Smyrk [23], who stated that a shift toward right-sided cancers has occurred during the second half of the twentieth century and Fenoglio-Preiser *et al.* [24] who stated that in low-risk countries, carcinomas of the cecum and ascending colon occur more frequent than left sided ones.

Regarding the gross pattern of tumors in the current study 42% were ulcerating, 38% were infiltrating and only 20% were fungating. This is compatible with what was reported by Day *et al.* [25], that most cancers of the colon and rectum are ulcerating tumors. In respect to histopathological types; 80% were adenocarcinoma, 20% were mucinous adenocarcinoma. These figures are near to what was reported by Lanza *et al.* [26], in which most colorectal carcinomas (85%) were adenocarcinomas and 10 to 15% were mucinous adenocarcinomas. Most CRC cases presented with positive lymph node metastasis (62%) and most cases (90%) presented with T3. According to the modified Duke's stage (Modified Astler–Coller) staging system, 58% of the cases were stage C and 38% were stage B and 4% were stage D. These results are not in agreement with those reported by Sis *et al.* [27], where the highest incidence was in stage B (46.4%). This may be explained by delayed onset of seeking medical advice or prevalence of right sided tumors in Egypt, which are usually diagnosed at a later onset. We observed tumor budding in 62% of cases and it showed a statistical significant correlation with advanced Duke's stage, positive lymph node metastasis and increasing stage of lymph node metastasis. Ohtsuki *et al.* [15] showed that budding was significantly associated with lymphatic vessel embolization at the invasive tumor margin, positive lymph node metastasis and local recurrence; this suggests that budding may occur during the initial phase nodal and distant metastasis. Guziñska-Ustymowicz [28] reported the positive relationship between tumor budding and expression of matrix metalloproteases; MMP-9, MMP-7, MMP-2 or Cathepsin B, that could explain the significant association between budding, depth of wall invasion and lymph node metastasis. Kye *et al.* [29] study was designed to identify risk factors for lymph node metastasis at early stage colorectal cancer, tumor budding (p=0.047) was the only factor that was significantly associated with lymph node metastasis in invasive T1 colorectal cancer. Ohtsuki *et al.* [15] compared budding assessment in HE-stained and immunohistochemically stained sections. Higher positivity for tumor budding was encountered with immunostaining with cytokeratin. Tanaka *et al.* [30] stated that immunohistochemical stains may be helpful in identification of tumor buds, especially if accompanied by an inflammatory reaction that obscures their presence.

The current study showed that 62% of cases showed low MVD (less than average MVD of the studied cases; 16.16) while 38% showed high MVD. The microvascular density only showed significant association with the sex of patient, as female cases showed higher MVD than male cases (P = 0.029), but no significant correlation was detected with the other clinicpathological parameters including tumor budding. Salim *et al.* [31] studied active angiogenesis by CD34 in CRC; they demonstrated that CD34 expression had no significant association with the clinicopathologic findings. This suggests that angiogenesis in CRC is not influenced by the degree of tumor grade and metastasis [32]. This finding was

previously demonstrated by Zhao *et al.* [33] on Chinese patients, Carneiro *et al.* [34] on Brazilian patients and Afrem *et al.* [35] on Romanian patients with CRC. In contrast, Giatromanolaki *et al.* [36] and Lazari et al [32] in their study on patients with CRC and gastric carcinoma respectively, demonstrated a significant correlation of high MVD with advanced stages. Another study done by Sharifi *et al.* [37] on Iranian patients, found a significant correlation between the MVD and different grades. Microvascular density may be of importance in planning for adjuvant and anti-angiogenic therapy after surgery [38].

In conclusion, the statistically significant correlation regarding tumor budding with advanced tumor stage, positive lymph node metastasis and increasing stage of lymph node metastasis in colorectal carcinoma cases supports the hypothesis that tumor budding acts a prognostic marker to predict poor outcome of patients with colorectal carcinomas. The study suggests that tumor budding might play a role in the ability of colon cancer cells to invade and metastasize, thus proper assessment of tumor budding in early stages of carcinoma may predict the ongoing strategy of surgical treatment, to avoid aggressive surgery in budding negative cases. According to these, it is recommended that a scoring system can be applied for tumor budding in CRC cases, to be reported in the patient's pathology report and could be useful in predicting the outcome in these patients. In order to elucidate the possible prognostic significance of MVD in colorectal carcinoma, it will be necessary to carry out similar studies on a larger sample size.

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