Immunohistochemical Expression of "HCG-β" in Colorectal Carcinoma

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Abstract

BACKGROUND: Tumor budding is associated with adverse histology and is a predictor of lymph node metastasis. Human chorionic gonadotropin-beta (hCG-β) expression in non-trophoblastic tumors has been associated with aggressive behavior.

AIM: Evaluation of tumor budding and hCG-β immunohistochemical expression in colorectal carcinoma (CRC), and correlation of their expression with various clinicopathological parameters.

MATERIALS AND METHODS: Immunohistochemical staining for hCG-β was performed on paraffin-embedded sections of 60 cases of CRC. Tumors with cytoplasmic or membranous staining of more than five epithelial cell clusters were designated hCG-β positive; otherwise, they were designated hCG-β negative. Tumor budding was assessed in hematoxylin and eosin stained slides and was classified as; low: 0–4 buds, intermediate: 5–9 buds and high: ≥10 buds; with exclusion of pure mucoid or signet ring cell morphology cases from analysis.

RESULTS: Tumor budding was low in (58.8%) of the cases, intermediate in (15.7%), and high in (25.5%). There was a statistically significant correlation between tumor budding and tumor histological grade (p = 0.011), lymph node metastasis (N) (p = 0.009), overall pathologic stage group (p = 0.009), modified Dukes' stage (p = 0.009), lymphovascular invasion (p = 0.000), and desmoplastic reaction (p = 0.004). Positive hCG-β alpha expression was detected in 12 (20%) of cases. There were statistically significant correlations between hCG-β expression and each of lymphovascular invasion (p = 0.042) and tumor budding (p = 0.000).

CONCLUSION: hCG-β is a marker of aggressiveness that may have essential role in tumor invasion. Tumor budding is crucial event in tumor invasion and metastasis. Tumor budding with hCG-β expression is a novel prognostic parameter and may represent a potential therapeutic target.

Introduction

Colorectal cancer is a major cause of morbidity and mortality throughout the world. Geographically, economically developed countries have the higher rates of colorectal carcinoma (CRC) [1]. Globally, CRC is the third most commonly diagnosed cancer in males and the second in females, while according to cancer deaths, CRC is the fourth leading cause in males and the third in females [2], while in Egypt, CRC accounts for 6.48% of all cancers according to the National Cancer Institute Cairo University [3].

Colorectal cancer development is a complex and heterogeneous process arising from interaction between multiple etiological factors, including genetic factors and environmental factors, for example diet and lifestyle [4].

Tumor-node metastasis (TNM) stage is the most important prognostic indicator for CRC. However, TNM stage does not take into account other features which allow for risk stratification, one of which is tumor budding [5].

Tumor budding is defined as a single tumor cell or a cell cluster of up to four tumor cells at the invasive front of the primary tumor and is detected in about 20–40% of colorectal cancer patients. Tumor budding is considered as a histologic finding in which tumor cells detach from the invasive margin of the tumor and migrate into the stroma surrounding the tumor, as well as a histomorphologic feature representing tumor aggressiveness [6].

Tumor budding is considered to be the first step in metastasis and has been associated with lymph node metastasis, distant metastasis, and increased risk of relapse [5]. Tumor budding is related to high tumor grade, infiltrating tumor border, lymphovascular invasion, and perineural invasion; therefore, many studies have suggested that tumor budding is an independent poor prognostic factor [6].

The initial step of invasion and metastasis in the process of tumor progression is epithelial-to-mesenchymal transition (EMT), and several transcription factors for example Snail, Slug, and Twist are involved. They decrease E-cadherin expression and increase fibronectin and N-cadherin expression, leading to a mesenchymal phenotype. Similar molecular findings are

noticed in tumor budding suggest that tumor budding is a form of EMT [5]. The EMT is characterized by loss of cell adhesion molecule E-cadherin, cytoskeletal alterations, increased production of extracellular matrix components, resistance to apoptosis, and ability to degrade basement membrane, resulting in a phenotype with increased migratory capacity, and invasiveness [7], [8]. In addition, tumor buds express matrix metalloproteinases, cyclin D1, vascular endothelial growth factor (VEGF) and p16, but do not show increased proliferation as determined by MIB1. Also of interest is that tumor buds may express stem cell markers, such as EpCAM [8].

Human chorionic gonadotropin (hCG) is a glycoprotein that consists of two polypeptide subunits (α and β) and constitutes five independent molecules in the body, including three α-β dimers and two β monomers. Free hCG-β is regarded as the specific and active subunit among the five different molecules. It increases spectacularly in the early stage of pregnancy and the majority of trophoblastic diseases has therefore been recognized as an excellent marker for both pregnancy and germ cell tumors [9]. It is also secreted in non-trophoblastic malignancies such as colorectal, gastric, and pancreatic carcinomas [5].

hCG-β secreting tumors are more aggressive, radio resistant and have a greater tendency to metastasize. Transforming growth factor β (TGF-β) is a major directing molecule in EMT in CRC through the TGF-β signaling pathway. Intriguingly, hCG-β shows significant homology with TGF-β [10]. Kawamata *et al.* propose the following mechanism for EMT in CRC with hCG-β overexpression; Tumor cells secrete hCG-β, which act through an autocrine fashion at the invasive front through TGF-β receptors. Binding of hCG-β to the TGF-β receptor activates downstream cascades and leads to altered transcription of EMT-related proteins. The tumor cells then acquire a mesenchymal phenotype and form tumor buds, which is capable of invasion and metastasis [10], [5].

Surgical resection combined with adjuvant therapy is an effective treatment at early stage of CRC, although subsequent relapse and metastasis often occur, also, at an advanced stage, resistance to conventional therapies is frequent and the existent treatments are ineffective. Therefore, a better understanding of the molecular mechanisms underlying the response to these drugs is essential for the identification of new predictive biomarkers and the development of targeted treatments [10].

Tumor budding and hCG-β expression are closely associated with EMT, and may act as molecular targets in CRC treatment [5]. hCG-β positive cells was mostly located at the invasive front of CRC tumors, the more malignant and aggressive tumor area; while hCG-β had no influence on tumor proliferation, it did promote CRC cells migration and invasion through EMT [9].

In colorectal cancer, hCG-β expression was shown to correlate with poor prognosis of CRC patients, but until now, the specific hCG-β role in CRC is unclear [9].

Materials and Methods

Retrieval of cases

The material of this cross-sectional study was collected as 60 formalin fixed, paraffin embedded tumor sections from colectomy specimens of patients with CRC, including tumor invasive fronts, from Kasr El Ainy Hospital, Faculty of Medicine, Cairo University and multiple private laboratories, in the time period from January 2019 to June 2019. The authors obtained the approval of ethical committee in the Faculty of Medicine, Cairo University.

Inclusion criteria included cases of CRC that underwent colectomy, including tumor invasive fronts. Exclusion criteria included cases received neoadjuvant therapy, and cases with deficient data.

The site of the tumor was classified into right-sided and left-sided as several studies have demonstrated that right- and left-sided colon cancers are genetically distinct [11]. Site was obtained from the pathology reports of the patients.

The tumor extension into the colonic wall and the lymph node status was obtained from the data in the final pathology reports.

Histopathological Examination

Each paraffin block was re-cut by rotatory microtome at 4 μM thickness then mounted on glass slides and stained by hematoxylin and eosin (H and E) for routine histopathological examination and on charged slides for immunostaining.

Morphologic classification of the colorectal cancer according to the recommendations of the World Health Organization [12] including histological types, tumor grade, depth of tumor invasion, perineural invasion, and lymphovascular emboli, while staging was performed using modified Dukes' classification of the disease [13] and the TNM staging system for each case. The TNM staging was applied according to the AJCC and the UICC where it encodes the extent of the primary tumor (T), regional lymph nodes (N), and distant metastases (M) [14].

In addition, tumor budding, desmoplastic reaction (DR), and Crohn-like lymphoid reaction (CLR) were assessed in the H and E stained slides. Tumor budding was scored according to the International Tumor Budding Consensus Conference (ITBCC) 2016 recommendations;

H and E-stained sections were scanned at medium power (10×) to identify the most dense area of budding at the tumor invasive front ("hotspot"). Tumor buds (defined as single tumor cells or clusters of up to 4 tumor cells) were counted in single field (20× magnification) in this area. The bud count was divided by the normalization factor (1.000) relative to the specific microscope eyepiece field number diameter (20 mm) to determine the tumor bud count per 0.785 mm^2 (fixed diameter was needed for assessment of tumor budding). The final bud count and the budding category (low: 0–4 buds [Figure 1a], intermediate: 5–9 buds [Figure 1b], high: 10 buds or more [Figure 1c]) were recorded. The procedure of tumor bud count is summarized in (Figure 2). Cases with pure mucoid or signet ring cell morphology were excluded from analysis [15].

The DR was histologically classified as one of three categories (mature, intermediate, or immature) based on the existence of keloid-like collagen or myxoid stroma in the reactive fibrous zone at the advancing edge of the tumor. Keloid-like collagen comprised broad bundles of hypocellular collagen with the brightly eosinophilic hyalinization typically observed in a keloid. Myxoid stroma can be defined as an amorphous stromal substance composed of an amphophilic or slightly basophilic material usually intermingled with randomly oriented keloid-like collagen. DR was regarded as mature when fibrotic stroma did not include keloid-like collagen or myxoid stroma and was formed of fine mature collagen fibers stratified into multiple layers (Figure 1j). When keloid-like collagens were intermingled in mature stroma, typically having parallel orientation to the mature collagen fibers, the fibrotic stroma was designated as undergoing intermediate maturation (Figure 1k). Fibrotic stroma with myxoid changes was considered as immature stroma (Figure 1l). The stromal assessment in each case was classified according to the most immature stromal area. Single microscopic field of a 40× objective lens of myxoid stroma was regarded as the minimum amount of myxoid stroma needed to be judged as immature stroma. The stroma around microscopic abscess was excluded from the assessment [16], [17].

CLR is nodular lymphoid aggregates (LAs) lining the tumor periphery in the deep portion of the bowel wall beyond invasive fronts of CRC, mainly in the muscularis propria and pericolic fat. The largest LA in each patient was identified, and its maximum diameter was measured. Ueno criteria depend on the largest LA size-based assessment; the CLR status is active when the maximum diameter of the largest LA ≥1 mm (Figures 1h and i), while inactive CLR when the maximum diameter of the largest LA <1 mm [16], [18].

hCG-β Immunohistochemical staining and evaluation

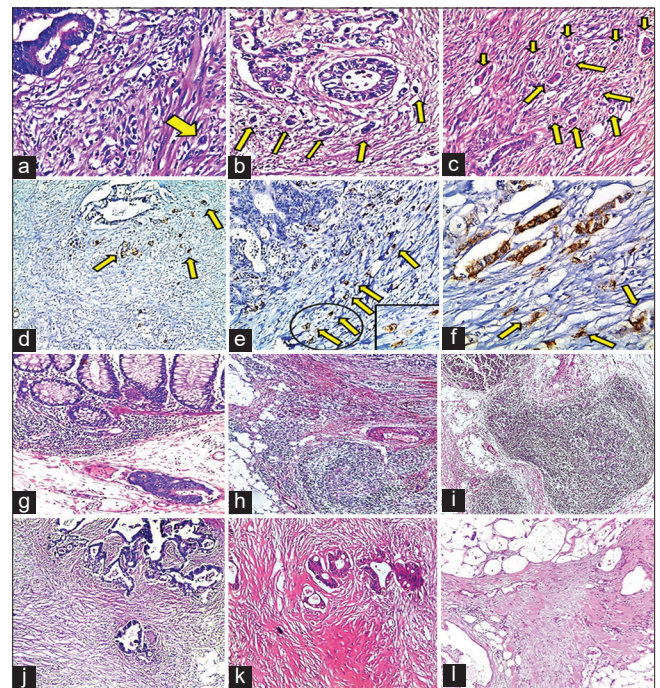A serial section (4 μM) from all cases was mounted on adhesive coated glass slides for hCG-β

Figure 1: (a-c); Adenocarcinoma GII with low [1 tumor bud], intermediate [5 tumor buds]; and high [>10 tumor buds] tumor budding, respectively, at invasive fronts (arrows point at tumor buds) (hematoxylin and eosin [H and E]; (a) ×400, (b) ×200 and (c) ×200 original magnification). Figures (d-f); Positive hCG-β cytoplasmic expression in tumor buds (arrows) (IHC; (d) ×100, (e) ×100 and (f) ×400 original magnification) [tumor buds magnified at insert in figure (e). Figure (g); lymphovascular invasion in submucosa (H and E; ×200 original magnification). Figures (h and i); Active Crohn-like lymphoid reaction (H and E; ×40 original magnification). Figures (j-l); Mature, intermediate and immature desmoplastic reaction, respectively, (H and E; (j) ×100, (k) ×200 and (l) ×100 original magnification)

staining. Primary antibody included is ready-to-use polyclonal rabbit antibody against isolated beta-chain of hCG immunogen (code IR508, Dako, Denamrk). The Biotin based DAKO Envision system (DAKO Envision labeled polymer, peroxidase) was used as the detection system. The sections were incubated with the antibody for 60 min at room temperature. Positive control was placental tissue.

All assessments were made on viable tumor at 40× magnification. Tumors with cytoplasmic or membranous staining of more than five epithelial cell clusters were designated hCG-β positive; otherwise, they were designated hCG-β negative [5], [10].

Statistical analysis

Data were coded and entered using the IBM SPSS Statistics version 26. Data were summarized using mean and standard deviation for quantitative variables and frequencies (number of cases) and relative frequencies (percentages) for categorical variables. Comparison between two groups was done using unpaired t-student test. For comparing categorical data, Chi-square (χ^2) test was performed. Exact test was used instead when the expected

frequency is <5. p < 0.05 was considered as statistically significant.

Results

This study included 60 cases of CRC. The mean age in this study was 53.8 years (ranged between 27 and 75 years). Regarding the gender, there was female predominance (63.3%). Concerning the tumor site; 51.7% were right sided and 48.3% were left sided.

The most common histological type in the present study was adenocarcinoma (85%), while mucoid carcinoma was only 15%. According to the grade of differentiation, 3.3% were Grade I, 68.3% were Grade II, and 28.3% were Grade III. Regarding tumor invasion (T) in this study, most of the cases were T3 representing (73.3%). Concerning lymph node status (N), slightly more than half of cases (58.3%) showed lymph node metastasis (total N1 and N2) and 41.7% were N0. Stage grouping of TNM was applied, where Stage III was the most common (58.3%). According to modified Dukes' classification, Group C2 accounted for the highest percentage (56.6%).

Lymphovascular invasion (Figure 1g) and perineural invasion were detected in 65% and 41.7% of the cases respectively. Regarding DR, it was classified into mature, intermediate, and immature stroma with percentages 73.34%, 23.34%, and 3.32%, respectively. CLR was active in 18.3% and inactive in 81.7% of the cases.

Regarding tumor budding, although several different tumor budding scoring methods have been proposed in the literature, the standardized method as proposed by the ITBCC consensus recommendations was the applied one in this study as it has been included as an additional reporting parameter in the protocol of the College of American Pathologists (CAP). Mucoid carcinoma cases (nine cases) were excluded from tumor budding assessment. The cases were classified into low, intermediate, and high tumor budding with percentages 58.8%, 15.7%, and 25.5%, respectively. The pathological characteristics of the studied cases are summarized in (Table 1) and were presented in (Figure 1).

Expression of hCG-β was cytoplasmic. hCG-β stained sections are divided into; negative group (48) cases (80%) and positive group (12) cases (20%) (Figure 1).

Tumor budding showed statistically significant correlation with tumor histological grade (p = 0.011), lymph node metastasis (N) (p = 0.009), overall pathologic stage group (p = 0.009), modified Dukes' stage (p = 0.009), lymphovascular invasion (p = 0.000), and DR (p = 0.004). Higher rate of tumor budding was noticed with higher tumor invasiveness (T), but yet with no statistically significant correlation. Correlation of

Table 1: Pathological characteristics of the studied cases

Pathological characteristics	Number (%)
Sex	
Female	38 (63.3)
Male	22 (36.7)
Tumor site	
Right	31 (51.7)
Left	29 (48.3)
Histological type	
Adenocarcinoma	51 (85)
Mucoid carcinoma	9 (15)
Histopathological grade	
Grade 1	2 (3.3)
Grade 2	41 (68.3)
Grade 3	17 (28.3)
Extent of primary tumor (T)	
T1	0 (0)
T2	4 (6.7)
T3	44 (73.3)
T4	12 (20)
Lymph node status (N)	
N0	25 (41.7)
N1	19 (31.7)
N2	16 (26.6)
Lymphovascular invasion	
Absent	21 (35)
Present	39 (65)
Perineural invasion	
Absent	35 (58.3)
Present	25 (41.7)
Stage group	
I	3 (5)
II	22 (36.7)
III	35 (58.3)
Modified duke's stage	
A	0 (0)
B	
B1	3 (5)
B2	22 (36.7)
C	
C1	1 (1.7)
C2	34 (56.6)
D	0 (0)
Desmoplastic reaction	
Mature	44 (73.34)
Intermediate	14 (23.34)
Immature	2 (3.32)
Crohn-like lymphoid reaction	
Inactive	49 (81.7)
Active	11 (18.3)
Tumor budding	
Low	30 (58.8)
Intermediate	8 (15.7)
High	13 (25.5)

tumor budding with various pathological characteristics among studied cases is summarized in Table 2.

hCG-β expression showed statistically significant correlations with lymphovascular invasion (p = 0.042) and tumor budding (p = 0.000), indicating that positive hCG-β expression is associated with presence of lymphovascular invasion and higher rates of tumor budding (Figures 1d-f). Higher frequency of hCG-β expression was associated with higher tumor histological grade, higher tumor invasiveness (T), higher lymph node status (N), higher TNM pathologic stage, and higher modified Dukes' stage, but yet with no statistical significance. Correlation of hCG-β expression with various pathological characteristics among studied cases is summarized in Table 2.

Discussion

Globally, CRC is the third most commonly diagnosed cancer in males and the second in females,

Table 2: Correlation of tumor budding and hCG-β expression with various pathological characteristics among the studied cases

Pathological characteristics	Tumor budding				hCG-β expression		
	Low	Intermediate	High	p-value	hCG-β -ve	hCG-β +ve	p-value
Sex							
Male	-	-	-	-	19 (86.4)	3 (13.6)	0.507
Female	-	-	-		29 (76.3)	9 (23.7)	
Site							
Right	14 (56)	4 (16)	7 (28)	0.925	26 (83.9)	5 (16.1)	0.527
Left	16 (61.5)	4 (15.4)	6 (23.1)		22 (75.9)	7 (24.1)	
Histological grade							
Grade 1	1 (50)	1 (50)	0 (0)	0.011*	2 (100)	0 (0)	0.142
Grade 2	27 (65.9)	3 (7.3)	11 (26.8)		30 (73.2)	11 (26.8)	
Grade 3	2 (25)	4 (50)	2 (25)		16 (94.1)	1 (5.9)	
Histological type							
Adenocarcinoma	-	-	-	-	39 (76.5)	12 (23.5)	0.182
Mucoid carcinoma					9 (100)	0 (0)	
Extent of primary tumor (T)							
T1 and T2	4 (100)	0 (0)	0 (0)	0.925	4 (100)	0 (0)	0.574
T3 andT4	26 (55.3)	8 (17)	13 (27.7)		44 (78.6)	12 (21.4)	
Lymph node status (N)							
N0	17 (77.3)	0 (0)	5 (22.7)	0.009*	21 (84)	4 (16)	0.745
N1 and N2	13 (44.8)	8 (27.6)	8 (27.6)		27 (77.1)	8 (22.9)	
Lymphovascular invasion							
Absent	17 (94.4)	0 (0)	1 (5.6)	0.000*	20 (95.2)	1 (4.8)	0.042*
Present	13 (39.4)	8 (24.2)	12 (36.4)		28 (71.8)	11 (28.2)	
Perineural invasion							
Absent	21 (67.7)	4 (12.9)	6 (19.4)	0.289	30 (85.7)	5 (14.3)	0.210
Present	9 (45)	4 (20)	7 (35)		18 (72)	7 (28)	
Stage group							
I and II	17 (77.3)	0 (0)	5 (22.7)	0.009*	21 (84)	4 (16)	0.745
III	13 (44.8)	8 (27.6)	8 (27.6)		27 (77.1)	8 (22.9)	
IV	0 (0)	0 (0)	0 (0)		0 (0)	0 (0)	
Modified duke's							
A	0 (0)	0 (0)	0 (0)	0.009*	0 (0)	0 (0)	0.745
B	17 (77.3)	0 (0)	5 (22.7)		21 (84)	4 (16)	
C	13 (44.8)	8 (27.6)	8 (27.6)		27 (77.1)	8 (22.9)	
D	0 (0)	0 (0)	0 (0)		0 (0)	0 (0)	
Desmoplastic reaction							
Mature	29 (69.1)	5 (11.9)	8 (19)	0.004*	35 (79.5)	9 (20.5)	1
Intermediate	1 (11.1)	3 (33.3)	5 (55.6)		11 (78.6)	3 (21.4)	
Immature	0 (0)	0 (0)	0 (0)		2 (100)	0 (0)	
Crohn-like lymphoid reaction							
Inactive	24 (58.5)	5 (12.2)	12 (29.3)	0.262	39 (79.6)	10 (20.4)	1
Active	6 (60)	3 (30)	1 (10)		9 (81.8)	2 (18.2)	
Tumor budding							
Low	-	-	-	-	30 (100)	0 (0)	0.000*
Intermediate					5 (62.5)	3 (37.5)	
High					4 (30.8)	9 (69.2)	

*Statistically significant

while according to cancer deaths, CRC is the fourth leading cause in males and the third in females [2]. In Egypt, CRC accounts for 6.48 % of all cancers according to the National Cancer Institute Cairo University [19].

Although the classic TNM classification is used for the staging of CRC patients and selection for specific treatment, it is not a completely adequate method as patients at the same stage may have different clinical outcomes, thus unable to precisely predict prognoses. Therefore, it is needed to identify the molecular markers of more aggressive CRC to select patients for adjuvant systemic or targeted therapies [20]. Nevertheless, TNM stage does not take into account other features which allow for risk stratification, such as tumor budding [5].

Tumor budding is considered to be the first step in metastasis and has been associated with lymph node metastasis, distant metastasis, and increased risk of relapse [5]. Many studies have suggested that tumor budding is an independent poor prognostic factor because it is related to high tumor grade, infiltrating tumor border, lymphovascular invasion, and perineural invasion [6].

Free hCG-β is regarded as the specific and active subunit of hCG. It increases strikingly in the early stage of pregnancy and the majority of trophoblastic diseases have therefore been recognized as an excellent marker for both pregnancy and germ cell tumors [9]. It is also secreted in non-trophoblastic malignancies including colorectal, gastric, and pancreatic carcinomas [5].

hCG-β secreting tumors are more aggressive, radio resistant and have a greater propensity to metastasize. Interestingly, hCG-β shows significant homology with TGF-β, a major driving molecule in EMT in CRC via the TGF-β signaling pathway [10].

Tumor budding and hCG-β expression are closely associated with EMT, and may serve as molecular targets in CRC treatment [5].

Konishi *et al.* proposed that tumor budding with hCG-β expression is a novel prognostic marker in CRC. If pathological evaluation of the surgical specimen revealed both tumor budding and hCG-β expression, these patients may require more aggressive treatment. Moreover, if both tumor budding and hCG-β expression are detected in an endoscopically resected specimen, they may indicate a latent risk for lymph node metastasis, with the requirement of lymph node dissection even if endoscopic resection was complete [5].

Regarding tumor budding, although several different tumor budding scoring methods have been proposed in the literature, the standardized method as

proposed by the ITBCC consensus recommendations was the applied one in this study as it has been included as an additional reporting parameter in the protocol of the CAP. In the current study, cases were classified into low, intermediate and high tumor budding with percentages 58.8%, 15.7%, and 25.5%, respectively, while in Oh *et al.*, 2018, and Dawson *et al.*, 2019, studies, low tumor budding was 54% and 39.3%, intermediate tumor budding was 31.3% and 26.6%, and high tumor budding was 14.7% and 34.1%, respectively [6], [15].

In the current study, there were statistically significant correlations between tumor budding and some of the clinicopathological parameters; tumor histological grade, lymph node status (N), TNM pathologic stage, modified Dukes' stage, lymphovascular invasion, and DR, while there were statistically insignificant correlations between tumor budding with primary site of the tumor, tumor invasiveness (T), perineural invasion, and CLR. In general, the histopathological parameters of all studied cases pointed to more advanced local disease, metastasis, and thus more aggressive behavior in association with tumor budding.

The relationship between the site of the primary tumor and tumor budding in the present study was statistically insignificant (p = 0.925), which was consistent with the results of Konishi *et al.*, 2018, Eriksen *et al.*, 2018, and Dawson *et al.*, 2019 (p = 1, 0.105, and 0.9655, respectively) [5], [15], [21].

The tumor histological grade was statistically significantly correlated with tumor budding in the current study (p = 0.011), which was in agreement with results reported by Eriksen *et al.*, 2018 and Dawson *et al.*, 2019 (p = 0.001 and <0.002, respectively) indicating that high tumor grade is associated with higher rates of tumor budding [15], [21]. This is mostly attributed to less tendency of malignant cells to form glands and higher dis-cohesion in high grade tumors. The same findings yet with statistically insignificant correlations were reported by Konishi *et al.*, 2018 and Ustymowicz, 2018 studies (p = 0.064 and 0.458, respectively) [5], [22].

Regarding local tumor invasiveness (T) in this study, higher rate of tumor budding was present in T3-T4 cases and absent in T1-T2 cases but this results were statistically insignificant (p = 0.925), similar to results reported by Konishi *et al.*, 2018 study (p = 0.091) [5]. On the other hand, statistically significant correlation was reported by Ustymowicz, 2018; Şirin *et al.*, 2019 and Dawson *et al.*, 2019, studies between T and tumor budding (p = 0.001, 0.002 and <0.0001, respectively) [15], [22], [23].

In the current study, lymph node status (N) was statistically significantly correlated with tumor budding (p = 0.009), which was consistent with the results of El Sheikh *et al.*, 2016; Konishi *et al.*, 2018; Ustymowicz, 2018; Şirin *et al.*, 2019; and Dawson *et al.*, 2019, indicating that higher rates of tumor budding are associated with lymph node metastasis

Figure 2: Procedure proposed by the International Tumor Budding Consensus Conference 2016 for reporting tumor budding in colorectal cancer in daily diagnostic practice [8]

(p = 0.001, 0.006, 0.000, <0.001, and <0.0001, respectively) [5], [15], [22], [23], [24]. This relationship points to the role of tumor budding in EMT and as precedent step to vascular invasion.

Regarding AJCC staging system, the present study documented it's statistically significant correlation with tumor budding (p = 0.009), which is in agreement with the results of Oh *et al.*, 2018; Konishi *et al.*, 2018; and Dawson *et al.*, 2019 studies, indicating that tumor budding is crucial in the tumor invasion and metastasis. (p = <0.001, 0.006, and <0.0001, respectively) [5], [6], [15].

In this study, modified Dukes' staging was statistically significantly correlated with tumor budding (p = 0.009), which was consistent with the results of El Sheikh *et al.*, 2016 study, indicating that tumor budding is involved in the tumor invasion and metastasis (p = 0.002) [24].

Lymphovascular invasion in the current study was statistically significantly correlated to tumor budding (p = 0.000), which was consistent with the results of Oh *et al.*, 2018; Konishi *et al.*, 2018; and Dawson *et al.*, 2019 studies (p < 0.05) who reported significant correlation of tumor budding with lymphatic invasion and vascular invasion separately [5], [6], [15], indicating that higher rate of tumor budding is associated

with presence of lymphovascular tumor invasion. This positive relation points to the possible high ability of tumor buds' cells for production of a variety of matrix proteloytic enzymes like MMP and Cathepsin-D that degrade ECM and basement membranes materials, as well as, production of angiogenic factors such as VEGF, laminin5γ2 and CD44 that promote angiogenesis, which is in agreement with what stated by Zlobec and Lugli, 2010, Mitrovic *et al.*, 2012, and Lugli *et al.*, 2017 studies [7], [8], [25].

The relationship between perineural invasion and tumor budding in the present study was statistically insignificant (p = 0.289). In contrast, Oh *et al.*, 2018 and Dawson *et al.*, 2019 studies reported statistically significant correlation (p = <0.001 and <0.0001, respectively), which may be related to different sample sizes [6], [15].

The CLR showed a non-significant correlation with tumor budding in the present study (p = 0.262), which is in agreement with Ueno *et al.*, 2013 study (p = 0.1587) [26]. However, in our study, higher rates of tumor budding were more observed with inactive CLR, this implies that tumor buds may have acquired some aggressive characters that enable them to evade immune surveillance.

DR was statistically significantly correlated with tumor budding in this study (p = 0.004), which was consistent with the results of Ueno *et al.*, 2015 (p ≤ 0.0001), indicating that higher rate of tumor budding is associated with less mature stroma, where ECM components are not well formed yet so facilitates local invasion of tumor cells with lower productions of ECM proteases [16].

Relationship between DR with tumor type was significantly correlated in this study (p = 0.000). This correlation was not considered in other comparative studies. DR was statistically significantly correlated with tumor grade in the current study (p = 0.000), which was in agreement with results reported by Ueno *et al.*, 2015 and Ueno *et al.*, 2018 (p = 0.0078 and 0.0002, respectively), indicating that maturity of DR decreases with the decrease of differentiation; which might be induced by tumor released factors that act in paracrine fashion [16], [17].

Higher rates of tumor budding are associated with other adverse histologic features such as higher histologic grade, higher lymph node status (N), higher TNM stage, higher modified Dukes' stage, presence of lymphovascular invasion, and less mature DR, which suggest considering high rate of tumor budding as an independent poor prognostic factor.

hCG-β was positively expressed in 20% of the cases in the present study, which was near to the results of Konishi *et al.*, 2018 and Kawamata *et al.*, 2018 studies who had the same results for hCG-β expression (16.3%) [5], [10]. While, Li *et al.*, 2018, had found higher rate of hCG-β positive expression (36.8%) [9].

In the current study, there were statistically significant correlations between hCG-β expression and lymphovascular invasion, as well as tumor budding, while there were statistically insignificant correlations between hCG-β expression and some of the clinicopathological parameters; sex, tumor size, primary site of the tumor, tumor histological type, tumor histological grade, tumor invasiveness (T), lymph node status (N), TNM pathologic stage, modified Dukes' stage, perineural invasion, CLR, and DR.

The median age for cases with positive hCG-β expression in the current study was 54.5 years with standard deviation 10.9 years, while Li *et al.*, 2018 study reported median age of positive cases was 65.28 years with standard deviation 11.9 years [9]. In addition, the mean age of positive cases in the present study was 55.7 years, while was 53.4 years for negative cases; this data was not considered in other comparative studies.

The sex of cases was not significantly correlated with hCG-β expression in this study (p = 0.507), which was in agreement with what was stated by Li *et al.*, 2018 (p = 0.84) [9].

The relationship between tumor size and hCG-β expression was statistically insignificant in the present study (p = 1.000). This correlation was not considered in other comparative studies.

The site of the primary tumor was not significantly correlated with hCG-β expression in this study (p = 0.527), which was consistent with the results of Konishi *et al.*, 2018 study (p = 1.000) [5].

The tumor histological type showed a non-significant correlation with hCG-β expression in the present study (p = 0.182). This correlation was not considered in other comparative studies.

Concerning the tumor histological grade, hCG-β expression was seen in moderately and poorly differentiated cases, and absent in well differentiated cases in the current study, but these results were statistically insignificant (p = 0.142). The same findings yet with statistically significant differences were reported by Konishi *et al.*, 2018 and Kawamata *et al.*, 2018 (p < 0.05 in both studies) [5], [10].

Regarding the tumor invasiveness (T) in the current study, hCG-β expression was only seen in T3-T4 cases and absent in T1-T2 cases; however, this results were statistically insignificant (p = 0.574). This agreed with Konishi *et al.*, 2018, and Kawamata *et al.*, 2018, who reported higher hCG-β expression in T3-T4 cases than in T1-T2 cases but with statistically insignificant correlation (p > 0.05 in both studies) [5], [10].

Concerning lymph node status (N) in this study, hCG-β expression was higher in presence of lymph node metastatic deposits (N1-N2) than in absence of lymph node metastasis (N0) but these results were

statistically insignificant (p = 0.745). The same findings yet with statistically significant differences were reported by Konishi *et al.*, 2018; Kawamata *et al.*, 2018 and Li *et al.*, 2018 (p < 0.05 in all studies) [5], [9], [10].

Regarding AJCC staging system, the present study documented higher hCG-β expression in Stage III cases than in Stage I-II cases; however, these results were statistically insignificant (p = 0.745). Konishi *et al.*, 2018, and Kawamata *et al.*, 2018, stated statistically significant correlation regarding this point (p < 0.05 in both studies) [5], [10].

Concerning modified Dukes' staging in the this study, hCG-β expression was higher in Dukes' (C) cases than in Dukes' (B) cases but this was statistically insignificant (p = 0.745). The same findings yet with statistically significant correlation were reported by Li *et al.*, 2018, study (p < 0.05) [9].

Lymphovascular invasion in the current study was statistically significantly correlated to hCG-β expression (p = 0.042), which was consistent with the results of Konishi *et al.*, 2018, and Kawamata *et al.*, 2018, who showed higher hCG-β expression in cases with lymphatic invasion and vascular invasion separately; however, Konishi *et al.*, 2018, and Kawamata *et al.*, 2018, showed statistically significant correlation with lymphatic invasion and insignificant correlation with vascular invasion suggesting higher role for hCG-β expression in lymphatic invasion rather than vascular invasion (p < 0.05 for lymphatic invasion and > 0.05 for vascular invasion). Either results point to the expected role of hCG-β in local and vascular invasion by binding to TGF-β receptor, leading to altered transcription of EMT-related proteins and inducing a mesenchymal phenotype of tumor cells [5], [10].

Perineural invasion in the present study was insignificantly correlated with hCG-β expression (p = 0.210). This correlation was not considered in other comparative studies.

In this study, tumor budding was statistically significantly correlated with hCG-β expression (p = 0.000), which was consistent with the results of Konishi *et al.*, 2018 study (p < 0.05), indicating that higher tumor budding was associated with hCG-β expression which suggest that hCG-β expression may play a significant role in the progression of CRC, and it is essential to asses tumor budding and hCGβ expression in both surgical specimen and endoscopically resected specimen, as their concomitant expression may require more aggressive treatment and lymph node dissection (even if endoscopic resection is complete) [5].

CLR and DR in the present study were insignificantly correlated with hCG-β expression (p = 1.000 for both). These correlations were not considered in other comparative studies.

hCGβ may be considered as a marker of aggressiveness as its expression is frequently associated with higher rate of tumor budding, as well as, presence of lymphovascular invasion, higher tumor invasiveness (T), higher lymph node status (N), higher TNM pathologic stage, and higher modified Dukes' stage, which suggest poorer prognosis for cases with hCG-β expression.

Despite the fact that the current study and comparable ones, reported more or less close figures of hCG-β expression positivity and extent of tumor budding; however, different sample sizes, variable grades and pathologic stages enrolled in these studies might explain the variation in results regarding correlations between tumor budding and hCG-β expression in CRC with other clinicopathological parameters.

Conclusion

Finally, we concluded that higher tumor budding is statistically significantly correlated with higher tumor grade, higher overall pathological stage, higher Modified Dukes' stage, lymphovascular invasion, positive nodal metastasis and less mature stroma, highlighting the importance of considering tumor budding as independent poor prognostic factor and indicating that tumor budding is crucial in the processes of tumor invasion and metastasis. hCG-β immunohistochemical expression is statistically significantly correlated with lymphovascular invasion and tumor budding suggesting that hCG-β is a marker of aggressiveness that may have essential role in tumor invasion. Tumor budding with hCG-β expression is a novel prognostic indicator in CRC that requires more aggressive treatment.

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