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Tumor budding and human chorionic gonadotropin-β expression correlate with unfavorable patient outcome in colorectal carcinoma

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Abstract

Tumor budding is thought to represent a manifestation of epithelial-to-mesenchymal transition (EMT) and it has been correlated with poor patient outcomes in colorectal cancer (CRC). Our group recently demonstrated that human chorionic gonadotropin-β (hCGβ) modulates EMT in CRC. In the current study, based on the likely relationships between tumor budding and hCGβ expression, we examined their clinicopathologic significance in CRC. Twenty-eight of 80 (35.0%) CRC showed tumor budding. Tumor budding significantly correlated with lymph node metastasis ($P < 0.01$), pathologic stage ($P < 0.01$), lymphatic invasion ($P = 0.044$), and vascular invasion ($P = 0.013$). Thirteen of 80 (16.3%) CRC were hCGβ positive on immunohistochemistry. More tumor buds were present in the hCGβ-positive cases ($P < 0.01$), and tumor budding was significantly correlated with hCGβ positivity ($P < 0.01$). Cases with both tumor budding and hCGβ expression had the poorest prognosis compared with all other groups ($P < 0.01$). In conclusion, tumor budding and hCGβ expression are closely associated with EMT, and they are independent prognostic factors in CRC. They identify patients with an "EMT phenotype" who may respond to targeted molecular therapies.

Keywords: Tumor budding・Human chorionic gonadotropin・Colorectal cancer・Epithelial-to-mesenchymal transition・Prognostic factor

Introduction

Colorectal cancer (CRC) is a major cause of death worldwide, accounting for 693,900 deaths in 2012 [1]. The most important prognostic indicator for CRC is tumor-node-metastasis (TNM) stage. However, TNM stage does not take into account other features which allow for risk stratification, one of which is tumor budding. Tumor budding is defined by the presence of individual tumor cells and/or small clusters of tumor cells at the invasive front [2]. Tumor budding is considered to be the first step in metastasis, as budding cells are thought to migrate through the extracellular matrix, invade lymphovascular structures and form metastatic deposits in lymph nodes at distant sites [3]. Indeed, tumor budding at the invasive front has been associated with lymph node metastasis, distant metastasis and increased risk of relapse [4-8]. In the process of tumor progression, epithelial-to-mesenchymal transition (EMT) is the initial step of invasion and metastasis, and several transcription factors such as Snail, Slug and Twist are involved. They decrease E-cadherin expression and increase N-cadherin and fibronectin expression, resulting in a mesenchymal phenotype [9, 10]. Similar molecular findings are seen in tumor budding [11, 12], suggest tumor budding is a form of EMT.

Human chorionic gonadotropin (hCG) is a glycoprotein hormone that consists of two polypeptide subunits (α and β) [13]. hCG has traditionally been used as a marker for pregnancy, pregnancy-associated disorders and gestational trophoblastic disease, but it is also secreted in non-trophoblastic malignancies including colorectal, gastric, and pancreatic carcinomas [14-16]. The ectopic production of the free β subunit, hCGβ, in the absence of the α subunit is a recognized phenomenon in the aforementioned epithelial tumors. According to several reports, hCGβ-secreting tumors are more aggressive, radioresistant, and have a greater propensity to metastasize [14, 17]. Of note, hCGβ shows significant homology with transforming growth factor β (TGFβ) [18], a major driving molecule in EMT, and we have recently shown hCGβ modulates EMT in CRC via the TGFβ signaling pathway [19].

Based on the likely relationships between tumor budding and hCGβ expression, we examined these features and their correlations with clinicopathologic parameters in 80 CRC.

Materials and methods

Human CRC samples

Eighty patients with invasive CRC who underwent radical surgery at Hokkaido University Hospital between 2002 and 2004 were included in the study. Patients were followed up at one- to six-month intervals until death or 31 December 2015, whichever was earlier. Patient clinical data are shown in Table 1. Approval was obtained from the Internal Review Board on Ethical Issues of Hokkaido University Hospital, Sapporo, Japan (14-005).

Pathologic analysis and immunohistochemistry

Formalin-fixed, paraffin embedded blocks from the 80 CRC were retrieved. One representative block containing invasive tumor was selected from each patient. From each block, one section was cut and stained with hematoxylin and eosin (H&E), and two sections were cut for pan-cytokeratin and hCGβ immunohistochemistry (IHC). Pathologic evaluation of primary tumor was performed according to the American Joint Committee on Cancer classification (7th edition) [20]. Other pathologic information (e.g. lymph node status) were obtained from the original pathology report.

Tumor budding was defined as isolated single cells or clusters of fewer than five cells [21]. Tumor budding was quantified on the pan-cytokeratin IHC section. Pan-cytokeratin IHC was performed using a mouse monoclonal antibody against pan-cytokeratin (AE1/AE3, 1:100; ACR011B; Biocare medical, Concord, USA). Budding number was determined by counting in the area of maximal budding, over one high-power field (HPF, 400×). Recently, high tumor budding (≧10) was reported to be an adverse prognostic factor for disease-free survival and overall survival in Stages I-III CRC [22]. Therefore tumors with more than 10

foci of budding per HPF were designated budding positive, otherwise they are designated budding negative.

hCGβ IHC was performed according to manufacturer instructions (Clone A0231, Dako, Tokyo, Japan). Briefly, after deparaffinization and rehydration, tissue sections were incubated with a rabbit polyclonal antibody against hCGβ (1:350; A0231; Dako, Tokyo, Japan), followed by reaction with a dextran polymer reagent combined with secondary antibodies and peroxidase (Envision/HRP; Dako, Tokyo, Japan). All assessments were made on viable tumor at 400× magnification. Tumors with cytoplasmic or membranous staining of more than five epithelial cell clusters were designated hCGβ positive [19], otherwise they were designated hCGβ negative.

Statistical analysis

We used χ^2 test or Fisher's exact test to examine correlations between tumor budding, hCGβ expression, and clinicopathological data. Survival curves for patients were constructed using the Kaplan-Meier method. Two-tailed Student's *t*-test was used to compare differences between two groups. Prognostic implications of tumor budding and hCGβ expression were analyzed by Cox univariate and multivariate proportional hazards models. The results were expressed as mean ± SD. Ekuseru-Toukei 2012 software for Windows was used for analysis (Social Survey Research Information Co. Ltd., Tokyo, Japan). Significance level was set at $P < 0.05$.

Results

Correlations between tumor budding, hCGβ expression and clinicopathologic parameters

The results are summarized in Table 2. Twenty-eight of 80 (35.0%) CRC were budding positive (Fig. 1a, 1b). Budding number ranged from 0 to 34 (mean ± SD, 8.9 ± 8.5). Tumor

budding was significantly correlated with lymph node metastasis (P = 0.006), pathologic stage (P = 0.006), lymphatic invasion (P = 0.044), and vascular invasion (P = 0.013). Thirteen of 80 (16.3%) CRC were hCGβ positive (Fig. 1c). hCGβ positivity significantly correlated with histologic grade (P = 0.031), lymph node metastasis (P = 0.021), pathologic stage (P = 0.021) and lymphatic invasion (P = 0.011). In all 13 hCGβ-positive CRC, hCGβ-expressing cells were present at the invasive front of the tumor (Fig. 1d), and in 10 out of 13 cases (76.9%), hCGβ expression was identified in tumor buds. Expression of hCGβ by tumor budding (Fig. 2a, 2b) was detected in 11 of 80 (13.8%) CRC and correlated with lymph node metastasis (P = 0.038) and pathologic stage (P = 0.038). Several studies have demonstrated that left- and right-sided colon cancers are genetically distinct [23, 24]. We therefore compared tumor budding and hCGβ expression with tumor location (left- and right-sided), but no significant differences were observed. Budding number was significantly higher in hCGβ-positive CRC (16.0 ± 8.2 vs 6.7 ± 7.2), and there was a significant correlation between tumor budding and hCG positivity (Table 3) (P < 0.01).

Correlations between tumor budding, hCGβ expression and patient survival

There was a trend that budding-positive CRC had an unfavorable prognosis compared with budding-negative CRC, although the result was not statistically significant (Fig. 2c). hCGβ-positive CRC had significantly poorer prognosis compared with hCGβ-negative CRC (Fig. 2d) (P = 0.02). CRC with both tumor budding and hCGβ expression had the poorest prognosis compared with all other groups (Fig. 2e) (P < 0.01). To further assess the significance of tumor budding and hCGβ expression, we performed univariate analysis using the Cox proportional hazards model. Vascular invasion, hCGβ expression, and tumor budding with hCGβ expression were significantly correlated with poor survival (Table 4). We then performed multivariate analysis to exclude influence by other factors. Tumor budding with hCGβ expression remained an independent predictor of poor survival (Table 4).

Discussion

In this study, we examined the clinicopathologic implications of tumor budding and hCGβ expression in CRC. Our findings suggest these two factors play a significant role in the progression of CRC, and there is an association between tumor budding and hCGβ expression at the invasive front. Notably, the group of patients showing both tumor budding and hCGβ expression had the poorest overall survival in comparison with the other groups. This group was more likely to have lymph node metastases and present at a higher pathologic stage. If this trend is confirmed with a larger patient cohort, tumor budding and hCGβ expression may be used as a prognostic marker in CRC.

Based on these results, we propose that tumor budding with hCGβ expression is a novel prognostic marker in CRC. If pathological evaluation of the surgical specimen reveals both tumor budding and hCGβ expression, these patients may require more aggressive treatment. Moreover, if both tumor budding and hCGβ 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 is complete.

The current consensus recommends tumor budding should be evaluated on H&E, since the vast majority outcome data are based on H&E assessment [25]. However, in some situations (e.g. marked peritumoral inflammation), tumor buds are difficult to identify among reactive stromal cells. In our study we therefore used both H&E and pan-cytokeratin IHC to visualize the tumor buds. It remains to be clarified whether tumor bud counts obtained via this approach have the same prognostic impact as tumor buds identified on H&E alone.

Although tumor budding may represent a manifestation of EMT, this hypothesis is not validated as the mechanisms by which budding cells detach from the main tumor are not clear. Previous gene expression studies have shown that the invasive front of CRC shows higher expression of Wnt/β-catenin target genes and upregulation of NF-κB target genes

compared with the tumor center [26, 27]. In a recent study, dissected budding cells are shown to display an EMT-like signature with activation of both TGFβ and Wnt signaling [28]. We recently showed hCGβ induces EMT through TGFβ receptor activation, and demonstrated the significance of TGFβ signaling in CRC [19]. Our current study further supports the hypothesis of a close relationship between tumor budding and EMT.

hCGβ expression in tumor budding represents a potential therapeutic target. In metastatic CRC, tumor budding correlates with resistance to epidermal growth factor receptor antagonists [29]. In a clinical trial, hCGβ vaccine considerably extended the survival of patients with advanced CRC [30]. The targeting of hCGβ-positive budding cells, whether by vaccines or recombinant antibodies, may be a therapeutic options for advanced CRC.

Our recent study was the first to suggest a relationship between hCGβ expression and EMT [19]. We showed that hCGβ-overexpressing CRC cell lines acquire a mesenchymal phenotype, demonstrate increased malignant potential compared with their control counterparts, and show altered expression of EMT-related proteins including E-cadherin, Snail, Twist and phospho-SMAD2. These changes result from activation of the TGFβ signaling pathway, since they are effectively reversed by TGFβ receptor inhibitors. Additional evidence exists for hCGβ signaling through the TGFβ pathway [31]. High levels of TGFβ and its receptor are commonly found in CRC [32, 33], hCGβ shows significant homology with TGFβ [18, 34], and the *TGFB1* gene (which encodes TGFβ) is located in close proximity to the *CGB* gene (which encodes hCGβ) [35].

We therefore propose the following mechanism [19]. Tumor cells secrete hCGβ, which acts in an autocrine fashion at the invasive front. Binding of hCGβ to the TGFβ receptor activates downstream cascades and leads to altered transcription of EMT-related proteins. This induces a morphologic change in the tumor cells, which acquire a mesenchymal phenotype and form tumor buds. The increased malignant potential of these cells allows for distant metastasis.

In conclusion, we have demonstrated that tumor budding and hCGβ expression occur commonly in CRC, and tumors showing both features have a poor prognosis. Tumor budding and hCGβ expression are closely associated with EMT, and may serve as molecular targets in CRC treatment.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standard of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the study.

Data availability: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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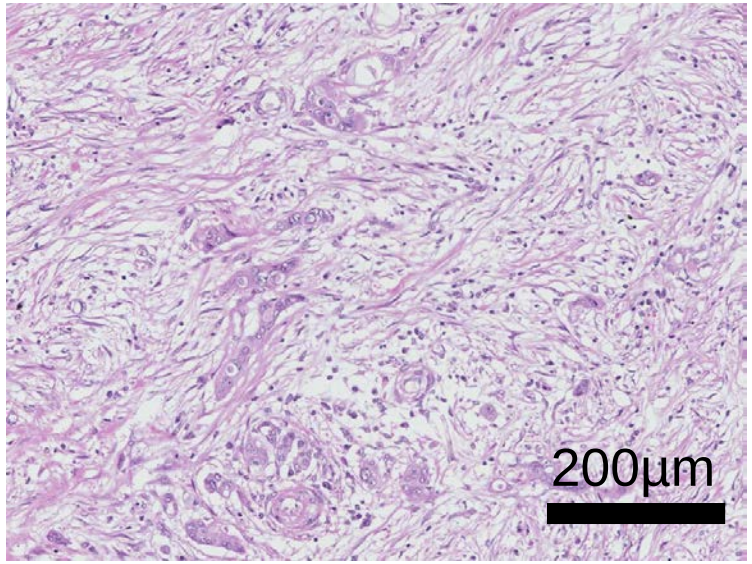
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Figure captions

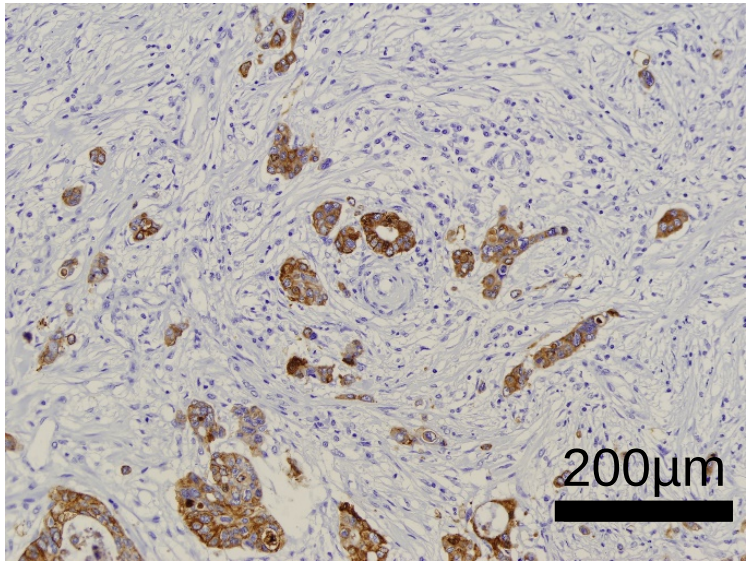
Fig 1 Representative examples of (a) H&E, (b) pan-cytokeratin immunohistochemistry, and (c) hCGβ immunohistochemistry. Budding cells are highlighted by pan-cytokeratin and, in this case, are also positive for hCGβ. (d) hCGβ-expressing cells are detected at the invasive front. Yellow allows indicate hCGβ-positive cells at invasive front

Fig 2 (a) Tumor budding within the muscularis propria (hCGβ immunohistochemistry). (b) A higher magnification view of a shows hCGβ expression within the budding cells. (c) Overall survival of tumor budding-positive patients did not differ significantly from tumor budding-negative patients. (d) Overall survival of hCGβ-positive patients was significantly poorer compared with hCGβ-negative patients. (e) Patients with both tumor budding and hCGβ expression had the poorest prognosis compared with all other expression groups

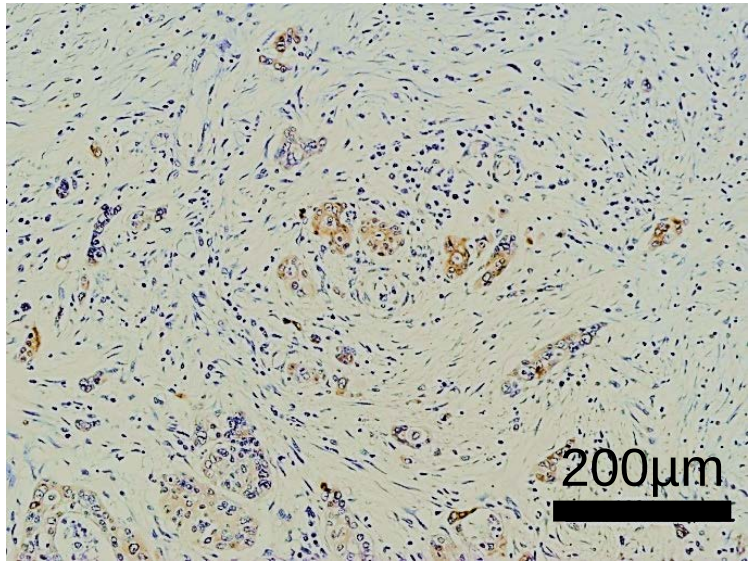
a H&E



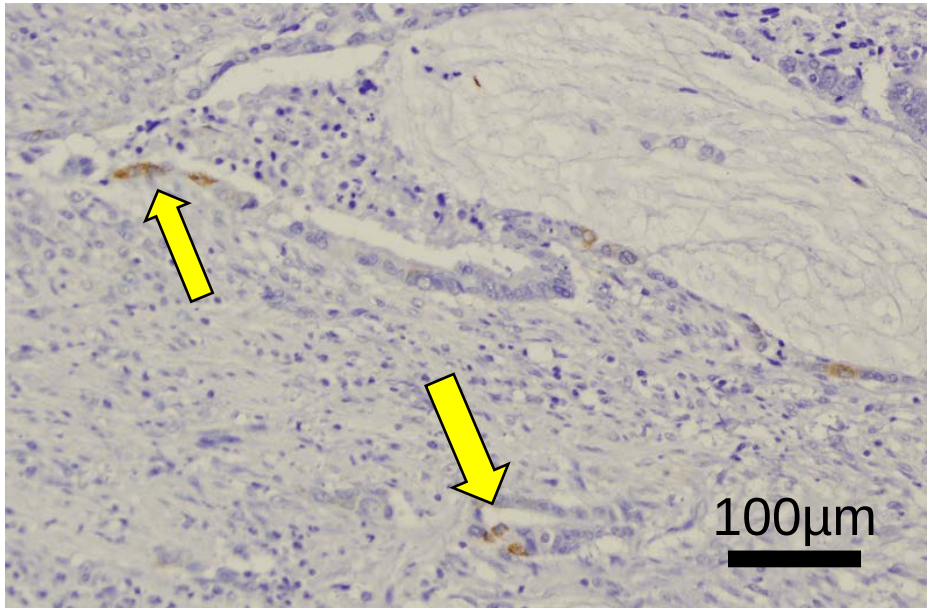
b AE1/AE3



c hCGβ

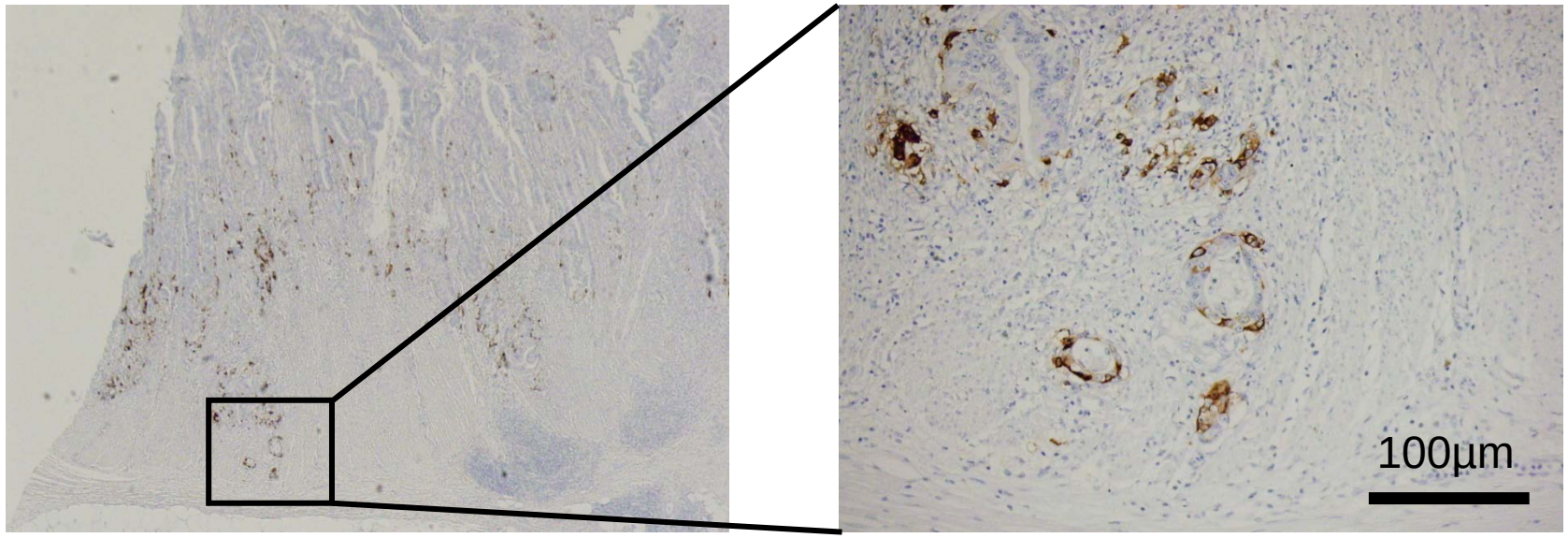


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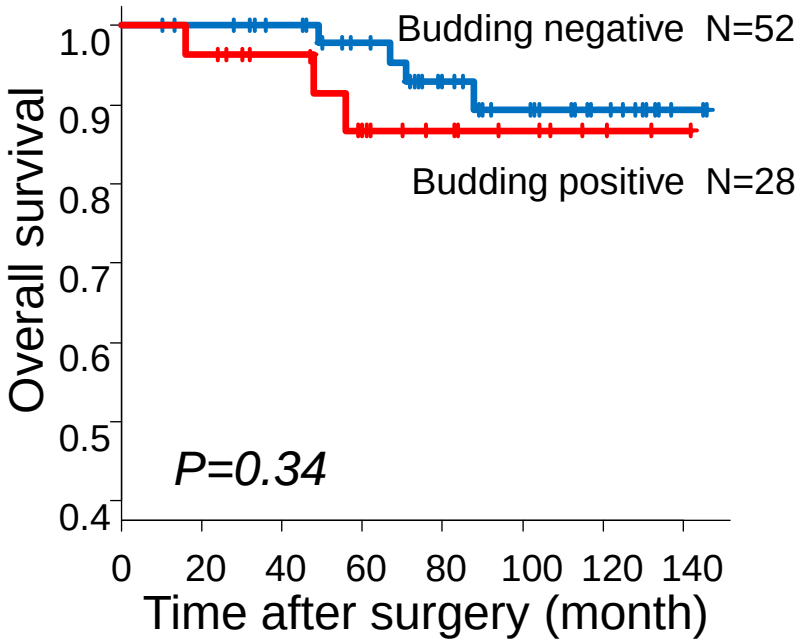


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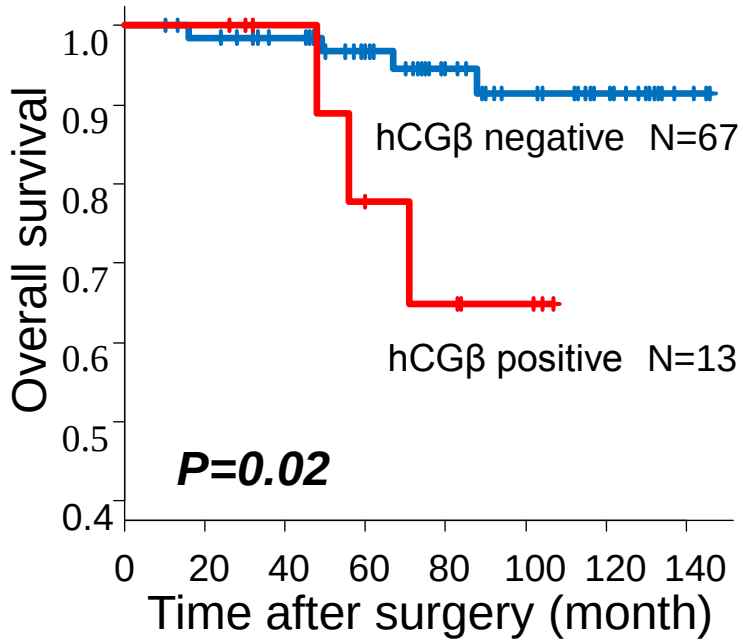
b


100μm

c


Tumor budding
Budding negative N=52
Budding positive N=28
P=0.34
Overall survival
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0 20 40 60 80 100 120 140
Time after surgery (month)

d


hCGβ expression
hCGβ negative N=67
hCGβ positive N=13
P=0.02
Overall survival
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0 20 40 60 80 100 120 140
Time after surgery (month)

e

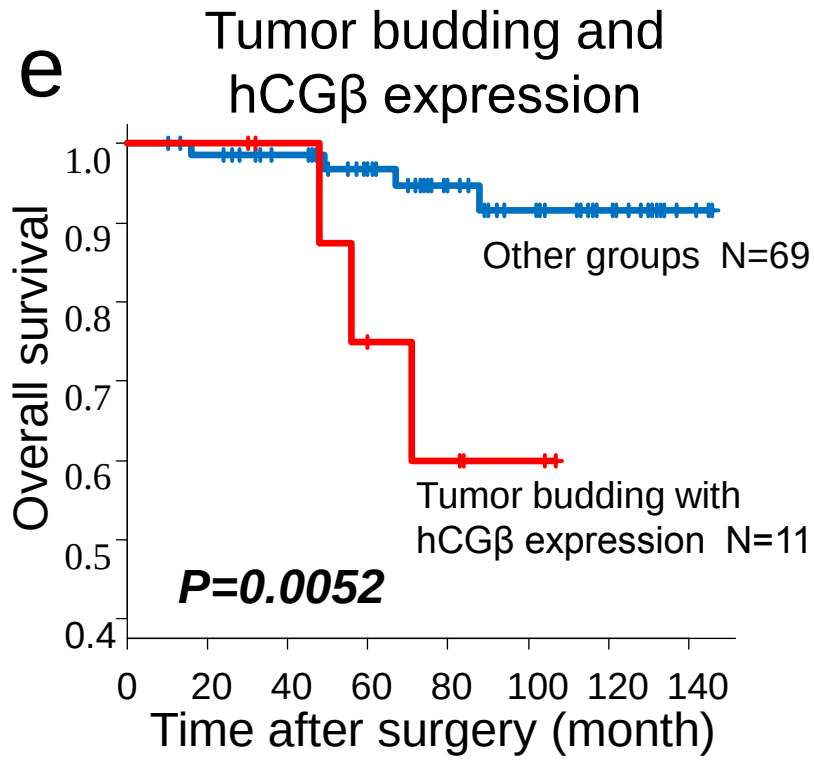

Tumor budding and
hCGβ expression
Other groups N=69
Tumor budding with
hCGβ expression N=11
P=0.0052
Overall survival
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0 20 40 60 80 100 120 140
Time after surgery (month)

Table 1 Patient clinicopathological characteristics

Parameter	No.
1. Age (years, mean ± SD)	65.2 ± 12.0
2. Gender	
Male	34
Female	46
3.Tumor location	
Right	29
Left	51
4. Histologic grade	
Well differentiated	34
Moderately/poorly differentiated	46
5. Resection status	
R0	79
R1	1
6. Pathologic T stage	
pT1-2	17
pT3-4	63
7. Pathologic N stage	
pN0	53
pN1-3	27
8. Overall pathologic stage	
I-II	53
III	27
9. Mean survival (months, mean ± SD)	79.0 ± 33.8

SD: Standard deviation

Table 2 Correlations between tumor budding, hCGβ expression and clinicopathologic parameters

Parameter	Total (n=80)	Tumor budding			hCGβ expression			Tumor budding and hCGβ expression		
		Positive (n=28)	Negative (n=52)	P value	Positive (n=13)	Negative (n=67)	P value	Yes (n=11)	No (n=69)	P value
1. Tumor location										
Right	29	10	19	1.000	6	23	0.531	5	24	0.515
Left	51	18	33		7	44		6	45	
2. Histologic grade										
Well differentiated	34	8	26	0.064	2	32	0.031*	2	32	0.106
Moderately/poorly differentiated	46	20	26		11	35		9	37	
3. pT stage										
pT1-2	17	3	14	0.091	1	16	0.192	1	16	0.441
pT3-4	63	25	38		12	51		10	53	
4. pN stage										
pN0	53	13	40	0.006*	5	48	0.021*	4	49	0.038*
pN1-3	27	15	12		8	19		7	20	
5. Overall pathologic stage										
Ⅰ-Ⅱ	53	13	40	0.006*	5	48	0.021*	4	49	0.038*
Ⅲ	27	15	12		8	19		7	20	
6. Lymphatic invasion										
Absent	38	9	29	0.044*	2	36	0.011*	2	36	0.051
Present	42	19	23		11	31		9	33	
7. Vascular invasion										
Absent	32	6	26	0.013*	4	28	0.458	3	29	0.511
Present	48	22	26		9	39		8	40	
8. Recurrence										
No	68	23	45	0.744	10	58	0.373	9	59	0.667
Yes	12	5	7		3	9		2	10	

* indicates statistically significant difference ($P < 0.05$)

Table 3 Correlation between tumor budding and hCGβ expression

	No.		
	hCGβ expression		
	Negative	Positive	Total
Tumor budding (mean ± SD)			
Negative (3.5 ± 3.3)	50	2	52
Positive (16.9 ± 7.0)	17	11	28
Total	67	13	80

χ^2/Fisher's exact test $P < 0.01$

Table 4 Univariate and multivariate analysis of patient survival in CRC

Parameter	Total (n=80)	Univariate analysis		Multivariate analysis		
		RR (95 % CI)	P value	RR (95 % CI)	Hazard ratio	P value
1. Tumor location		3.86 (0.89-1.39)	0.239		NC	
Right	29					
Left	51					
2. Histologic grade		3.92 (1.10-2.41)	0.113		NC	
Well differentiated	34					
Moderately/poorly differentiated	46					
3. Pathologic N stage		2.27 (1.31-1.94)	0.163		NC	
pN0	53					
pN1-3	27					
4. Overall pathologic stage		2.27 (1.31-1.94)	0.163		NC	
I-II	53					
III	27					
5. Lymphatic invasion		3.56 (0.59-1.19)	0.275		NC	
Absent	38					
Present	42					
6. Vascular invasion		4.37 (1.58-4.21)	0.040*****	0.95–13.18	3.53	0.353
Absent	32					
Present	48					
7. Tumor budding		1.89 (0.65-0.90)	0.343		NC	

No	52					
Yes	28					
8. hCGβ expression		6.12 (0.74-5.87)	0.015*	0.08-9.92	5.33	0.054
No	67					
Yes	13					
9. Tumor budding and hCGβ		6.26 (0.82-7.82)	0.005*	1.44-29.34	6.49	0.015*
No	69					
Yes	11					

* indicates statistically significant difference (P <0.05)

RR, relative risk/hazard ratio; CI, confidence interval; NC, not calculable.