Tumor budding in colorectal carcinoma assessed by cytokeratin immunostaning and budding areas: possible involvement of c-Met

Keisuke Satoh[1, 2], Satoshi Nimura[1], Makoto Hamasaki[1], Mikiko Aoki[1], Kaori Koga[1], Hiroshi Iwasaki[1], Yuichi Yamashita[2], Hiroaki Kataoka[3]and Kazuki Nabeshima[1]

[1]Department of Pathology, Fukuoka University School of Medicine and Hospital, Fukuoka, Japan; [2]Department of Gastroenterological Surgery, Fukuoka University School of Medicine and Hospital, Fukuoka, Japan and [3]Section of Oncopathology and Regenerative Biology, Department of Pathology, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan

Correspondence: Professor K. Nabeshima, Department of Pathology, Fukuoka University School of Medicine and Hospital, 7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan

E-mail: kaznabes@fukuoka-u.ac.jp

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Summary: Although tumor budding/sprouting has been shown to be an independent adverse prognostic factor in T1 and T3N0 colorectal carcinomas, its assessment could be improved by more accurate identification of budding carcinoma cells and consideration of budding areas. Moreover, tumor budding mechanisms are yet to be defined. In this study, we evaluated the identification of budding tumor cells by either HE staining alone or HE with immunohistochemistry and developed a scoring system based on budding grades and areas. We examined whether the budding score correlated with clinicopathologic features and prognosis and the association between tumor budding/sprouting and c-Met protein expression and phosphorylation and *MET* gene copy numbers because c-Met is known to play an important role in colorectal carcinoma tumorigenesis. Cytokeratin immunohistochemistry could identify tumors with shorter disease-free survival (DFS) from the low-grade budding group assessed with HE alone. High budding scores based on budding grade and area were more significantly correlated with DFS than scores obtained using the budding grade alone. In tumors with a high budding score c-Met expression and phosphorylation levels and *MET* gene copy numbers were significantly increased at the invasive front compared with those in superficial tumor portions. This study showed for the first time that high levels of phospho-c-Met at the invasive front were significantly associated with a high budding score and shorter DFS. In conclusion, a budding score assessed by budding grades and budding-positive areas correlates highly with clinicopathologic aggressive features of colorectal carcinoma.

Introduction

Colorectal carcinoma is one of the most common solid tumors worldwide.[1] Clinicopathologic staging of colorectal tumors is performed for prognosis and treatment decision using the tumor node metastasis (TNM) staging system from the Union for International Cancer Control.[2] However, a substantial number of tumors demonstrate local or distant recurrences despite being categorized as low risk by the TNM system.[3, 4] The failure of TNM staging to serve as a reliable prognostic system for patients with intermediate-stage tumors may be overcome by considering morphologic, molecular, or treatment-related factors that can stratify patients more precisely into different categories of risk.[5] Thus, the search for additional factors to assess the prognosis of colorectal carcinomas has been a major research focus. The promising histopathologic parameters in colorectal carcinoma include extramural venous invasion, tumor border configuration (pushing vs. infiltrating), presence of an inflammatory infiltrate, and tumor budding/sprouting, defined as small clusters of tumor cells at the invasive front.[3]

The concept of tumor budding/sprouting was first described in the 1950s by Imai, who postulated that the presence of 'sprouting' at the invasive edge of carcinomas reflected a more rapid tumor growth rate.[3] Although a large number of studies have shown that tumor budding/sprouting is an independent adverse prognostic factor in colorectal carcinoma, assessment methods and definitions have varied widely between studies.[3] It is sometimes difficult to detect budding foci by conventional pathological examination of hematoxylin-eosin (HE)-stained

sections alone because tumor buds can be very small and resemble the surrounding stromal cells.[3, 6] Furthermore, the distribution and frequency of budding foci appear differs even among tumors with the same budding grade.

The molecular mechanisms underlying tumor budding/sprouting remain unclear. In this study, we focused on the role of c-Met in budding tumor cells. The c-Met protein is an important factor in colorectal tumorigenesis, progression, and metastasis.[7] The *MET* gene is located on chromosome 7 at q31 and encodes a transmembrane glycoprotein that serves as a specific receptor for hepatocyte growth factor (HGF).[8] Binding of HGF to c-Met induces phosphorylation of tyrosine residues at the C-terminus of the receptor, leading to receptor activation.[9] HGF/MET signaling promotes multiple biological activities, including cell proliferation, motility, invasion, angiogenesis, and morphogenesis in a wide variety of normal and neoplastic cells.[10] Moreover, c-Met activity is deregulated in many human cancers, including colorectal carcinoma, as a result of genetic mutations, gene amplification, protein overexpression, or production of HGF-dependent autocrine circuits.[11, 12] In cororectal carcinoma, increased expression of the c-Met protein is associated with highly invasive tumors that spread through the intestinal wall.[8, 13]

Our study had two major aims: (1) To evaluate the associations between our scoring system for tumor budding/sprouting, which included budding grade and the proportion of budding-positive areas, and clinicopathologic factors or prognosis; and (2) To assess the association between c-Met expression and tumor budding/sprouting. Assessment of the budding score was significantly associated with lymphovascular invasion,

lymph node (LN) metastasis, and poor prognosis. Moreover, we found a significant correlation between c-Met expression levels at the invasive tumor front and budding score.

Materials and Methods

Patients. We retrospectively reviewed 139 patients who underwent surgical resection of primary colorectal adenocarcinomas at the Department of Gastroenterological Surgery, Fukuoka University Hospital, Japan from January 2005 to December 2009. Patients with familial adenomatous polyposis, hereditary non-polyposis colorectal cancer syndrome, or inflammatory bowel disease were excluded. Tissues from surgical resections can be used for research according to the standard treatment agreement with patients in our hospital, provided patient anonymity is maintained and the patient has no objections. The protocol for this study was approved by the Institutional Review Board (The Ethics Committee). Pathologic stage and tumor differentiation were determined by the TNM classification of malignant tumors (UICC) and Japanese Classification of Colorectal Carcinoma (JCCC),[14] respectively. Complete tumor resection was achieved in 114 cases, including 10 cases of pTis tumors for which endoscopic treatment could not be performed. None of the patients received preoperative radiotherapy or chemotherapy.

Tissue samples and immunohistochemistry (IHC). Surgically resected specimens were fixed in 10% formalin and processed into paraffin blocks. Tissues were sectioned (3-μm thickness), deparaffinized, and

immersed in 0.3% hydrogen peroxide in methanol for 10 min at room temperature (RT) to block endogenous peroxidase activity. For anti-cytokeratin (CK) antibody staining, sections were heated in 10 mM ethylenediaminetetraacetic acid (EDTA) buffer (pH 8.0) in a microwave oven (700 W) for 10 min to retrieve epitopes. After nonspecific sites were blocked with Serum-Free Protein Block (Dako, Carpinteria, CA) for 30 min at RT, sections were incubated with antibodies against CK (AE1/AE3) (dilution 1:200; Dako), c-Met (dilution 1:500; Santa Cruz Biotechnology, Santa Cruz, CA), or phosphorylated c-Met (p-c-Met) (dilution 1:1,000; Immuno-Biological Laboratories, Takasaki, Japan) for 1 hour at RT (AE1/AE3) or overnight at 4°C (c-Met, p-c-Met). Sections were then washed and incubated with ChemMate EnVision (Dako). Immunoreactive proteins were visualized with 3,3′-diamino-benzidine (DAB) (Dako), followed by counterstaining with hematoxylin. A diagnosis was independently made by two pathologists and confirmed when the two opinions were concordant.

Scoring system for tumor budding/sprouting. Based on JCCC,[14] we defined tumor budding/sprouting as a cancer cell nest consisting of five or fewer cells that infiltrated the interstitium at the invasive margin of the cancer (Fig. 1a, b). After selecting an area in which budding/sprouting was most intensive, the buds were counted in a field measuring 0.785 mm^2 through a 20×objective lens (WHK 10×ocular lens, Olympus). Depending on the number of buds, the criteria by JCCC for estimation of tumor budding/sprouting were modified as follows: Grade 0, no budding; Grade 1, 1-4 buds; Grade 2, 5-9 buds; Grade 3, 10 or more buds. We used this grading system to evaluate budding/sprouting in HE- and CK-immunostained sections. The extent of budding/sprouting was

classified as low grade (grades 0 and 1) or high grade (grades 2 and 3).

The distribution of budding foci differed, even among tumors of the same grade (Fig. 2a, b). For example, in some tumors only a few grade 2 budding/sprouting foci were present whereas other tumors showed grade 2 budding foci at higher frequencies along the invasive front. Therefore, we assessed both budding grade and the proportion of budding-positive areas. Each budding grade (grade 0-3 by CK-IHC) was multiplied by an area score (0.25 for 0–25%, 0.5 for 26–50%, 0.75 for 51–75%, and 1 for 76–100%), giving a maximum total score of 3. This budding score was classified as low (<1) or high (≥1).

Scoring for c-Met and p-c-Met. Immunoreactivity for c-Met or p-c-Met was observed as cytoplasmic or membrane staining in carcinoma cells. Staining results were assessed semi-quantitatively with the modified McCarty's H-scoring system.[15] The intensity score (0 for none, 1 for weak, 2 for moderate, and 3 for strong) and proportion score (0 for 0–5%, 1 for 6–25%, 2 for 26–50%, 3 for 51–75%, and 4 for 76–100%) were multiplied to yield an overall c-Met or p-c-Met score. These c-Met and p-c-Met scores were classified as low or high corresponding to overall scores of 0–4 or 6–12, respectively.

Fluorescent in situ hybridization (FISH) analysis. FISH assays were performed at the invasive fronts and within superficial portions of tumors for 21 or 22 randomly selected recent cases of budding/sprouting with low or high score, respectively. The *MET* gene was evaluated on formalin-fixed paraffin-embedded 4-μm thick tissue sections using Vysis LSI MET/CEP 7 probe (Abbott, Tokyo, Japan) with slight modifications as described

previously.[16] At least 60 cells were scored for each case.

The copy number of the *MET* gene was classified into six categories (disomy, low and high trisomy, low and high polysomy, and gene amplification) according to the number of copies of the *MET* gene and chromosome 7 centromere.[10] The mean *MET* gene copy number per cell was also recorded for each case.

Statistical analysis. The relationships between several clinicopathologic parameters and the results of budding assessment, FISH, and IHC were evaluated with the chi-square test and Fisher's exact test. The results of IHC and FISH analyses were compared using the Mann-Whitney U test at both the invasive front and superficial portions of colorectal cancers. Survival curves were plotted by the Kaplan-Meier method, and *P*-values were calculated with the log-rank test. A *P*-value <0.05 was considered statistically significant. All data analyses were conducted with StatMate IV for Windows (ATMS Co., Tokyo, Japan).

Results

Clinicopathologic findings and budding grades by HE staining or CK-IHC. Table 1 summarizes the clinicopathologic characteristics of the 139 patients (84 males and 55 females; age range, 25–94 [mean=66.7] years). The median follow-up period was 40.5 months (range, 0–93).

Tumor budding/sprouting was first assessed in HE-stained sections. However, assessment was sometimes difficult because of the presence of reactive fibroblasts or macrophages in the stroma surrounding the invasive

front, therefore we investigated whether it was easier to detect budding carcinoma cells with CK-IHC. Among 139 tumors, HE revealed low- and high-grade budding in 101 (72.7%) and 38 (27.3%) tumors, respectively, whereas CK-IHC showed low- and high-grade budding in 45 (32.4%) and 94 (67.6%) tumors, respectively (Fig. 1a-c). CK-IHC revealed approximately 2.5-fold more cases of high-grade budding than HE staining. Comparing the two assessment methods, 37 of 38 tumors that showed high-grade budding with HE remained high by CK-IHC (high-to-high group). In contrast, only 44 of 101 tumors that showed low-grade budding with HE remained low grade with CK-IHC (low-to-low group) and 57 were classified as high-grade (low-to-high group). Both HE and CK-IHC-based grades showed significant association with all three parameters, lymphatic and venous invasion and lymph node metastasis (Table 2). However, the high grade of the CK-IHC budding grade detected more cases with lymphatic and venous invasion and lymph node metastasis than that of the HE budding grade (80% vs 41%, 78% vs 34%, and 96% vs 54%, respectively), indicating usefulness of the CK-IHC grade. When disease-free survival (DFS) was assessed in 113 patients who received curative resections (40 low-to-low, 47 low-to-high, and 26 high-to-high), the low-to-high group (blue line) showed an intermediate survival curve between that for the low-to-low (green line) and high-to-high (red line) groups (Fig. 1d). The differences between low-to-low and low-to-high and between low-to-low and high-to-high groups were statistically significant. The presence of the low-to-high group, which showed survival in between the low-to-low and high-to-high group, also supported the clinicopathological significance of the CK-IHC grade.

Scoring for tumor budding/sprouting. Next, we considered budding-positive areas in our assessment of tumor budding/sprouting as described in the Materials and Methods section (Fig. 2a, b) and identified 51 (36.7%) low-score and 88 (63.3%) high-score tumors. A high score for budding-positive areas was significantly associated with greater wall penetration, increased lymphovascular invasion, and more frequent lymph node metastasis (Table 3).

The association between budding score or grade and DFS was assessed in 114 patients who received curative resections. DFS was significantly shorter in patients with high budding scores or grades compared with that in patients with low budding scores or grades, respectively ($P<0.001$ for budding scores and $P=0.002$ for budding grade) (Fig. 2c). Thus, both budding score and grade revealed significant association with DFS. In this study, we employed budding score for analyses of budding mechanisms since tumor budding is assessed more precisely by budding grade and areas, although budding grade alone is more practical for daily diagnosis.

c-Met and p-c-Met scores at different tumor sites. We examined the relationship between the expression of c-Met or p-c-Met and the extent of tumor budding/sprouting to assess the role of c-Met signaling in tumor budding/sprouting. Carcinoma cells including budding cells expressed c-Met and p-c-Met in the cytoplasm and cell membrane, as reported in other tumors. [17, 18]Representative images of different intensity scores for c-Met and p-c-Met are shown in Figure 3.

Tumors with both low and high scores for tumor budding/sprouting showed significantly higher c-Met

scores at the invasive front than in superficial portions of the tumors. Moreover, c-Met scores at the invasive front were significantly higher in tumors with high budding scores than those in tumors with low budding scores ($P<0.05$). In contrast, c-Met scores in superficial portions were not significantly different between tumors with low and high scores for tumor budding/sprouting ($P=0.32$) (Fig. 4a).

IHC for p-c-Met showed similar results as staining for c-Met, except that p-c-Met scores were not significantly different between the invasive front and superficial portions of tumors with low-score budding (Fig. 4b). Tumor cells expressing c-Met and p-c-Met were observed more frequently in budding cells and tumor cell nests at or near the invasion front than superficial portions of tumors with high budding scores. This difference in distribution of p-c-Met expressing cells was not evident in tumors with low budding scores.

FISH analysis. FISH for *MET* copy number demonstrated occasional trisomy, polysomy, and disomy (Fig. 5a). Amplification was not observed in this study (n=43 tumors). The *MET* gene copy number per cell was significantly higher at the invasive front than in the superficial portion of tumors in tumors with a high budding score. However, the copy number was less than 2.5 even at the invasive front of tumors with high-score budding, therefore no gene amplification was detected (Fig. 5b).

Univariate analysis. Among 114 tumors, univariate analysis identified significant associations between shorter DFS and budding score (high score), budding grades (high grades), lymphatic invasion (positive), venous invasion (positive), LN metastasis (positive), tumor size (≥5cm), pathologic wall penetration (T3 and T4), p-c-Met

score (high score), and *MET* gene copy number per cell (≥2.4) (Table 4).

Discussion

This study has shown that tumor budding/sprouting assessed by grade and area shows a strong correlation with clinicopathologically aggressive parameters in colorectal carcinoma. Although our assessment may not be useful for routine clinical practice, it is appropriate for examining the molecular mechanisms of tumor budding/sprouting. This is the first suggestion of a potential association between c-Met activation and formation of tumor budding/sprouting at the invasive front.

Morodomi et al.[19] and Ueno et al.[20] provided budding grades that can be used routinely for histopathologic diagnosis by examination of HE sections. However, these grades have several limitations regarding objective assessment of tumor budding/sprouting. In some cases, tumor buds cannot be detected easily with HE staining alone because stromal reaction, such as aggregation of active fibroblasts and/or histiocytes, is present at the invasive front and often masks the buds. Also, marked inflammation can obscure budding tumor cells.[3] Therefore, as in previous studies,[2, 6, 21-25] we assessed tumor budding/sprouting by IHC with anti-CK antibody to more accurately and objectively diagnose tumor budding/sprouting. We found that CK-IHC was very useful for the detection of single cancer cells or small cancer cell clusters embedded in fibrous stroma. One previous study[25] that included both HE and CK-IHC showed a modest improvement in inter-observer variability with the latter.[3] We

compared budding counts obtained with HE to those obtained with CK-IHC and obtained significantly higher counts with the latter. Moreover, CK-IHC could identify tumors with shorter DFS among tumors assessed as low-grade budding by HE alone. Thus, CK-IHC was useful for objective evaluation of budding grade.

In our study, the frequency of tumor budding/sprouting along the entire invasive front differed, even among tumors with the same budding grade. The majority of previous studies evaluated the budding grade only at the invasive front with the greatest amount of tumor budding/sprouting, although a few studies[26] also evaluated the proportion of budding-positive areas. The latest study by Horic et al[27] reported that one visual field is suboptimal to assess tumor budding because of heterogeneous biological features in CRC. Tumor buds are not homogeneously distributed along the invasive border. Therefore, Horic et al recommends that tumor budding assessment should be performed using 10-high-power-fields (10HPF) to account for the biological variation within a histological section. We assessed tumor budding variation in terms of the frequency of budding along the invasive border instead of assessing 10 HPF. In this way, we developed an original scoring system that includes both budding grade and area. A high budding score using our system was significantly correlated with a shorter DFS, and this correlation was slightly more significant than that obtained by assessing budding grade alone. These results suggest that the budding score is a predictive factor for recurrence after curative resection.

Previous studies have shown positive staining for c-Met by IHC in 60–79% of colorectal carcinomas[28, 29]

and *MET* gene amplification by FISH in 2.6% of carcinomas.[30] However, the relationship between c-Met and tumor budding/sprouting has not been explored fully. In this study, the expression and phosphorylation levels of c-Met were significantly higher at the invasive fronts than in superficial portions of tumors in the high-score budding group. No *MET* gene amplification was detected, although a significant increase in the *MET* gene copy number per cell was seen at the invasive front of the high-score budding group. Metastatic colon carcinoma tissues in the liver showed the gene copy number similar to that at the invasive front (n=3, 2.39±0.16, preliminary study, data not shown). However, the gene copy numbers were less than 2.5 in both metastatic liver tumors and invasive fronts of tumors with high-score budding, and thus their biological or clinical significance in c-Met expression is still unknown and needs to be clarified in the future. Therefore, in this study, we consider that post-transcriptional activation of c-Met by phosphorylation may be involved in tumor budding/sprouting at invasive fronts. This phosphorylation of c-Met may be induced by HGF produced by stromal cells. In colorectal carcinoma, HGF was actually detected in mesencymal cells in association with c-Met expression in carcinoma cells.[31] A previous study showed that c-Met was expressed in tumor cells and stromal HGF at a significantly higher level in type C invasive tumors than in type A and B non-invasive tumors based on Noguchi's classification for lung adenocarcinomas.[10] In another study using three-dimensional co-cultures of a ductal breast carcinoma *in situ* cell line and HGF-secreting fibroblasts, fibroblast-derived HGF promoted progression of *in situ* ductal carcinoma to invasive carcinoma.[32] These results indicate that HGF produced by active fibroblasts might be one of the factors

triggering the transition of non-invasive tumors to invasive adenocarcinomas. Therefore, the expression and phosphorylation of c-Met at tumor invasive fronts observed in our study may be related to tumor-stromal interactions. Furthermore, presence of more p-c-Met expressing cells at the invasion front in the high-score budding group suggested association of the activated c-Met signaling pathway with tumor budding/sprouting. Activation of c-Met in tumors leads to cell proliferation, angiogenesis, invasion and cell migration, including cell scattering.[10-12] The presence of tumor buds is considered to represent epithelial mesenchymal transition (EMT).[33] EMT is frequently associated with increased expressions of molecules relevant to tumor invasion, such as MMPs (matrix metalloproteinases), β-catenin, and laminin 5γ2, in tumor cells and stimulation of the Wnt signaling pathway.[33] In our study, p-c-Met expression was frequently observed not only in tumor buds but also in larger tumor nests near the invasion front, suggesting that an additional factor(s) may be needed for cells' undergoing tumor budding/sprouting. Our finding that the p-c-Met score was associated with postoperative recurrence is also of potential clinical significance. Future studies should examine whether c-Met inhibition reduces the invasiveness or recurrence of colorectal carcinoma.

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Disclosure statement

The authors declare no conflict of interest.

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Figure legends

Figure 1. HE staining of colorectal cancer showing tumor budding/sprouting (a) and cytokeratin immunohistochemistry (CK-IHC) for tumor budding/sprouting (b) at the invasive front. (c) Classification of budding grade by HE staining and CK-IHC. (d) Disease-free survival (DFS) curves for patients with different budding grades assessed by HE and CK-IHC. Green line, 40 tumors assessed as low grade budding with both HE and CK-IHC; blue line, 47 tumors assessed as low grade with HE but high grade with CK-IHC; red line, 26 tumors assessed as high grade with both HE and CK-IHC.

Figure 2. Score for budding/sprouting based on grade and area. (a, b) Both of these tumors were categorized as budding grade 2, but the area of the invasive front positive for tumor budding/sprouting was different. (c, d) Disease-free survival curves stratified by budding score and grade for 114 patients who underwent complete resection of colorectal tumors. (c) DFS curves for patients with high and low budding score were significantly different ($P<0.001$). (d) DFS curves for patients with high and low budding grade assessed by cytokeratin immunohistochemistry were significantly different ($P=0.002$).

Figure 3. Representative images of c-Met expression in tissue sections of colorectal cancer by

immunohistochemistry. (a) No c-Met expression in tumor cells (scored as 0). (b) Weak c-Met expression in tumor cells (scored as 1). (c) Moderate c-Met expression in tumor cells (scored as 2). (d) Strong c-Met expression in tumor cells forming nests near the invasion front (scored as 3). (e) No p-c-Met expression in tumor cells in superficial portion (scored as 0). (f) Moderate p-c-Met expression in tumor cells including budding cells at the invasive front (scored as 2).

Figure 4. Comparison of c-Met (a) and p-c-Met score (b) in different regions of the tumor between tumors with high and low budding score.

Figure 5. (a) Dual-color fluorescent in situ hybridization (FISH) assays with probes for *MET* (red) and chromosome 7 centromere (green). The *MET* gene copy number in the cells shown was two (arrow head) and four (arrow). (b) Comparison of *MET* gene copy number per cell by FISH in different regions of tumor between tumors with high and low budding score.

Table 1 Clinicopathological features

Patient	139	
Gender		
Male	84	(60.4%)
Female	55	(39.6%)
Age (year)		
Mean	66.7	
Range	25-94	
Location		
Cecum	8	(5.8%)
Ascending colon	21	(15.1%)
Transverse colon	15	(10.8%)
Descending colon	7	(5.0%)
Sigmoid colon	30	(21.6%)
Rectum	58	(41.7%)
Tumor differentiation		
Well	63	(45.3%)
Moderately	75	(54.0%)
Poorly	1	(0.7%)
Wall penetration (pT)		
pTis	10	(7.2%)
pT1	27	(19.4%)
pT2	21	(15.1%)
pT3	47	(33.8%)
pT4	34	(24.5%)
Lymphatic invasion		
Present	80	(57.6%)
Absent	59	(42.4%)
Venous invasion		
Present	103	(74.1%)
Absent	36	(25.9%)
Lymph node metastasis		
Present	50	(36.0%)
Absent	89	(64.0%)
TNM stage		
0	9	(6.5%)
I	42	(30.2%)
II	29	(20.9%)
III	35	(25.2%)
IV	24	(17.3%)

Table 2 Clinicopathological findings in relation to budding grade by HE staining and immunohistochemistry

	Budding grade (HE)			Budding grade (IHC)		
	Low grade	High grade	*P*-value	Low grade	High grade	*P*-value
Lymphatic invasion						
Present	47	33	<0.001	9	71	<0.001
Absent	54	5		36	23	
Venous invasion						
Present	68	35	<0.01	23	80	<0.001
Absent	33	3		22	14	
Lymph node metastasis						
Present	23	27	<0.001	2	48	<0.001
Absent	78	11		43	46	

The degree of budding/sprouting was classified as low grade or high grade corresponding to 0-4 (Grade 0 and Grade 1) and ≥5 budding foci (Grade 2 and Grade 3) in one field, respectively.

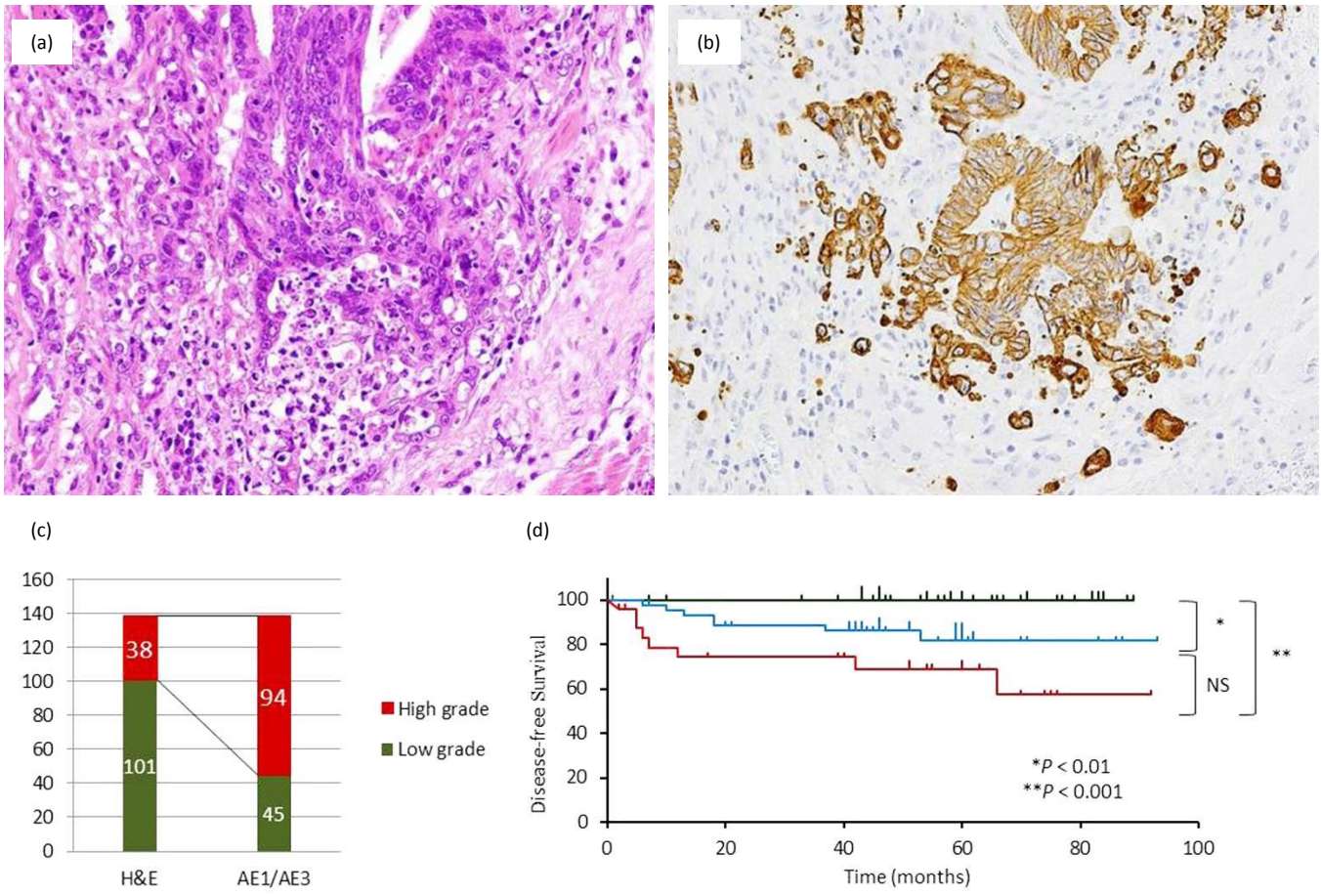
Table 3 Clinicopathological findings in relation to budding score

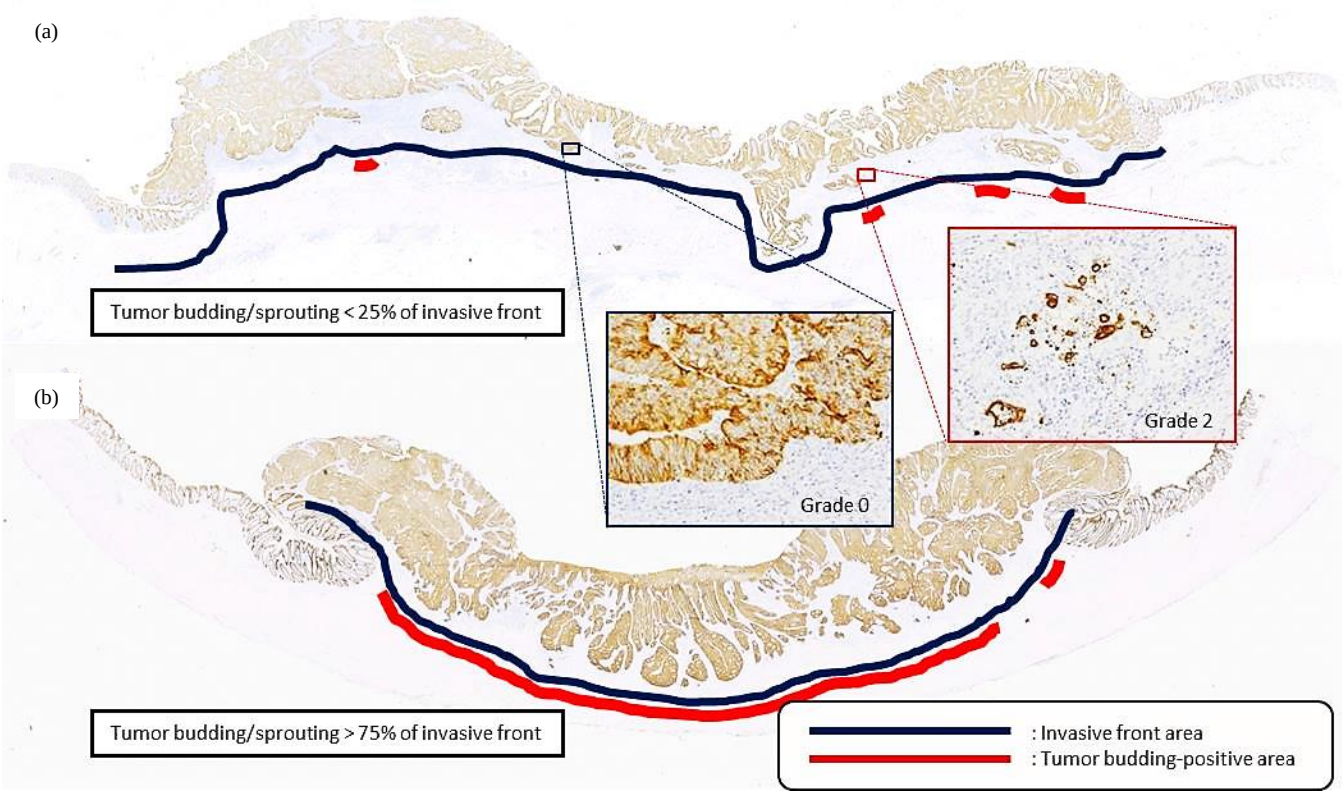
	Budding score		
	Low score (score <1) (n=51)	High score (score ≥1) (n=88)	*P*-value
Tumor size			
<5cm	38	59	0.36
≥5cm	13	29	
pT-stage			
pTis, pT1, pT2	33	25	<0.001
pT3, pT4	18	63	
Lymphatic invasion			
Present	14	66	<0.001
Absent	37	22	
Venous invasion			<0.001
Present	28	75	
Absent	23	13	
Lymph node metastasis			<0.001
Present	6	44	
Absent	45	44	

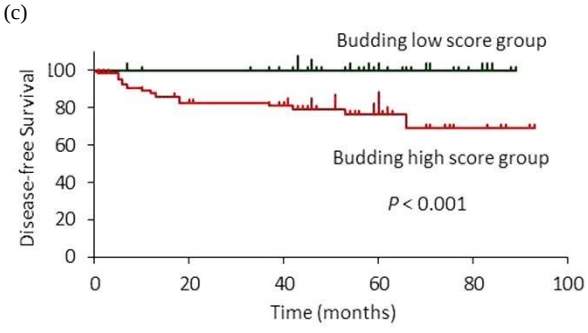
Table 4 Result of univariate analysis on postoperative disease-free survival

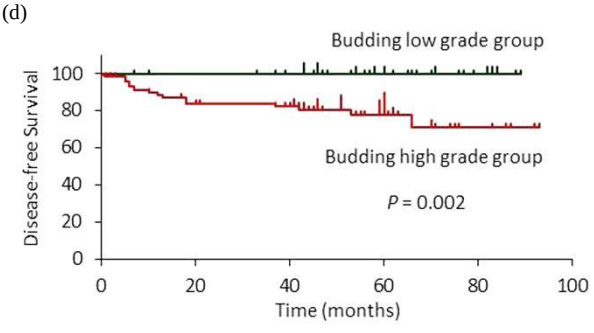
Factor		Disease-free Survival (n=114)	
		n	*P*-value
Budding grade (IHC)	Low grade	41	0.002
	High grade	73	
Budding score	Low score	46	<0.001
	High score	68	
Lymphatic invasion	Present	58	0.01
	Absent	56	
Venous invasion	Present	78	0.024
	Absent	36	
Lymph node metastasis	Present	35	0.006
	Absent	79	
Tumor size	<5cm	86	<0.001
	≥5cm	28	
Wall penetration (pT)	Tis, T1, T2	57	<0.001
	T3, T4	57	
cMet score	Low score	47	0.67
	High score	67	
p-cMet score	Low score	35	0.03
	High score	79	
MET gene copy number per cell	<2.4	24	0.14
	≥2.4	11	

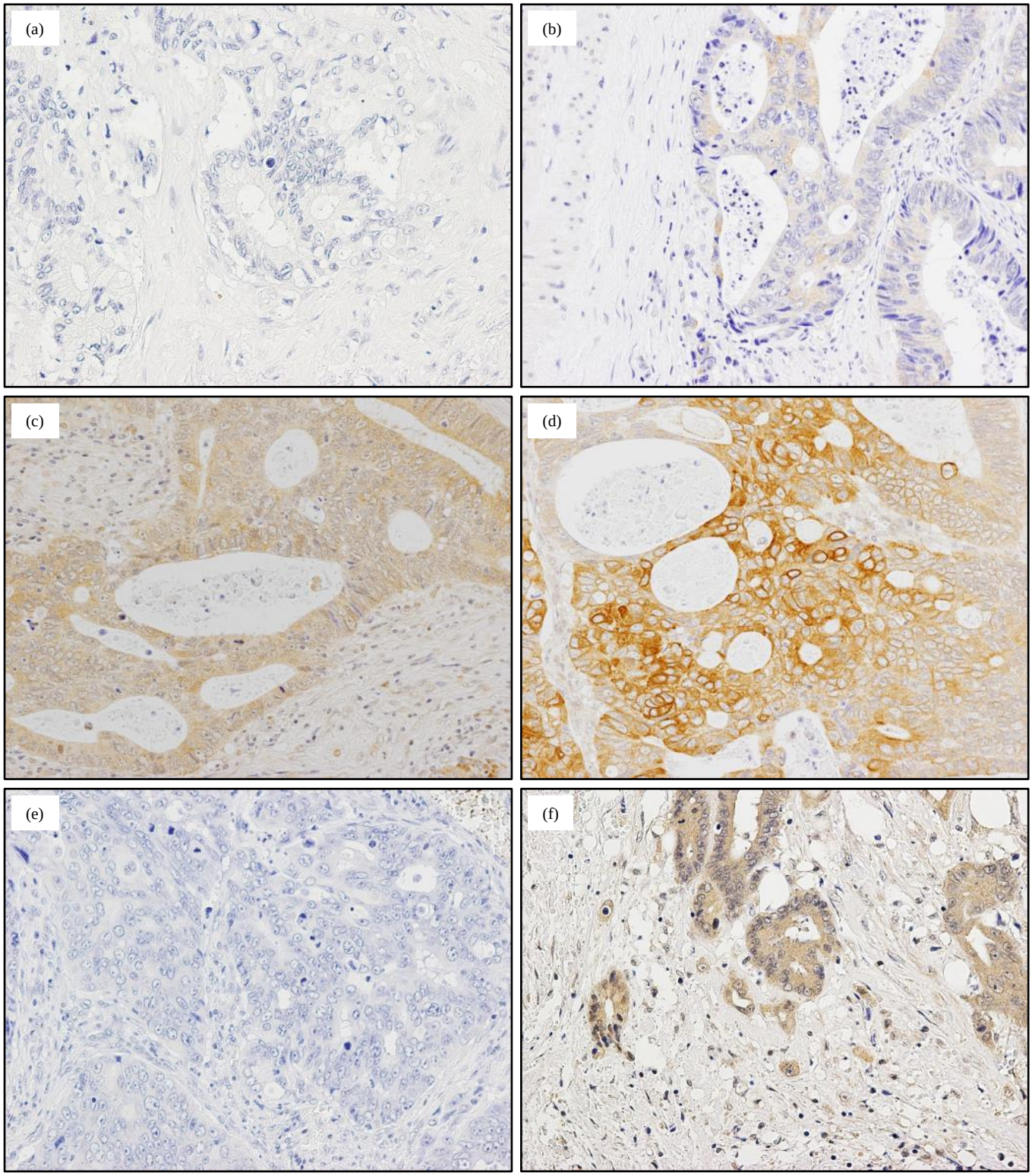
The score of cMet and p-cMet was classified as low score or high score corresponding to 0-4 and 6-12, respectively.

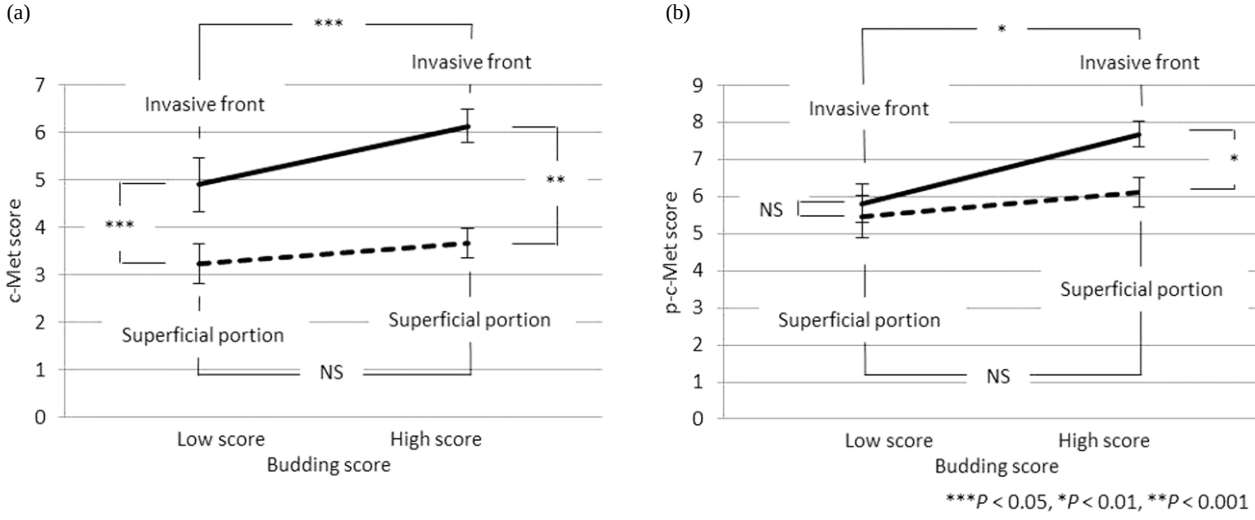

(a)
(b)
(c)
160
140
120
100
80
60
40
20
0
38
101
94
45
H&E
AE1/AE3
High grade
Low grade
(d)
Disease-free Survival
100
80
60
40
20
0
0
20
40
60
80
100
Time (months)
*
**
NS
*P < 0.01
**P < 0.001

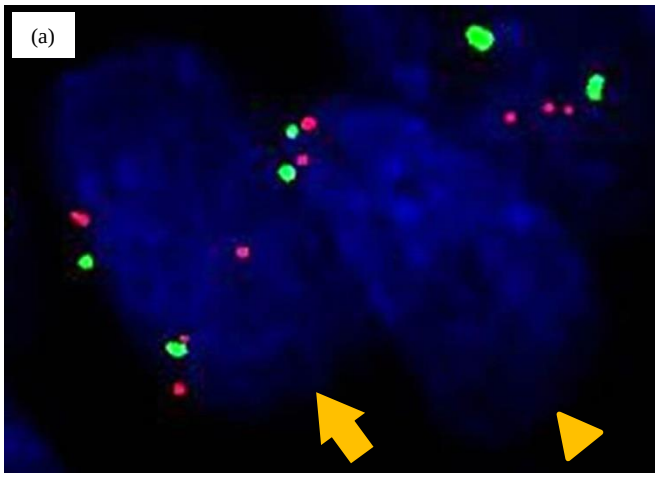

(a)
Tumor budding/sprouting < 25% of invasive front
Grade 0
Grade 2
(b)
Tumor budding/sprouting > 75% of invasive front
: Invasive front area
: Tumor budding-positive area

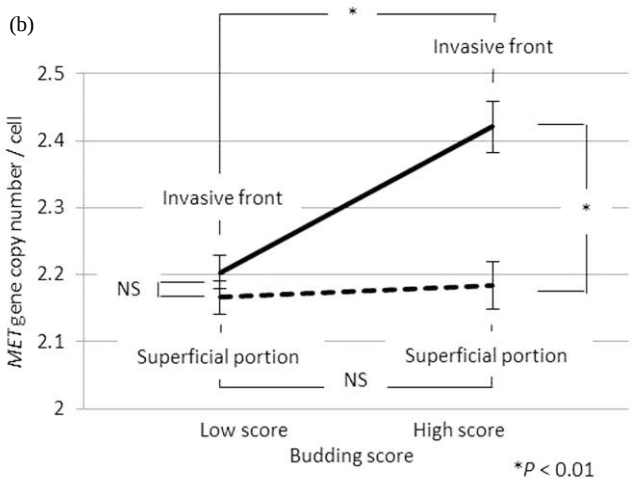

(c)
Budding low score group
Budding high score group
P < 0.001
Disease-free Survival
Time (months)
0
20
40
60
80
100


(d)
Budding low grade group
Budding high grade group
P = 0.002
Disease-free Survival
Time (months)
0
20
40
60
80
100


(a)
(b)
(c)
(d)
(e)
(f)


(a)
***
Invasive front
Invasive front
**
***
Superficial portion
Superficial portion
NS
c-Met score
0
1
2
3
4
5
6
7
Low score
High score
Budding score
(b)
*
Invasive front
Invasive front
*
NS
Superficial portion
Superficial portion
NS
p-c-Met score
0
1
2
3
4
5
6
7
8
9
Low score
High score
Budding score
***P < 0.05, *P < 0.01, **P < 0.001


(a)


(b)
*
Invasive front
2.5
2.4
2.3
2.2
2.1
2
MET gene copy number / cell
Invasive front
*
NS
Superficial portion
Superficial portion
NS
Low score
High score
Budding score
*P < 0.01