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Tumour Budding as an Independent Prognostic Factor for Survival in Patients With Distal Bile Duct Cancer

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Abstract. *Background/Aim: Surgical resection is the standard treatment for bile duct cancer. However, even when surgical resection is possible, the 5-year survival rate is reportedly 25.0-55.0%. Therefore, bile duct cancer is associated with poor prognoses. We conducted a clinicopathological investigation, focusing on the histological phenomenon of tumour budding, which has previously been reported to be correlated with the survival of patients with a variety of cancers. Patients and Methods: To investigate the significance of tumour budding in distal bile duct cancer, we recruited 65 patients who underwent pancreatoduodenectomy at our institution between 1995 and 2011. Tumour budding was observed and evaluated using the 'hot spot method'. The 'low' budding group comprised 0-4 cell clusters and the 'high' budding group ≥5 cell clusters. Additionally, immunostaining was performed in high-budding areas. Results: Tumour budding and stage were confirmed using a Cox proportional hazards model as independent prognostic factors for overall survival ($p<0.05$) in all patients. There was a significant association between budding and zinc finger E-box binding homeobox 1 expression, an endothelial-mesenchymal transition-induced transcription factor. In stage II cases, the prognosis was significantly worse in the 'high' budding group compared to that in the 'low' budding group. Conclusion: The budding phenomenon is an independent prognostic factor for patients with distal bile duct cancer. Understanding the mechanisms underlying tumour budding in distal bile duct cancer and its relationship with poor prognoses may be useful for the development of novel treatments for this disease.*

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Key Words: Bile duct cancer, budding phenomenon, EMT-induced transcription factors.

Bile duct cancer (BDC) is a condition associated with a poor prognosis, for which the standard treatment is surgical resection. Distal BDC (DBDC) often induces jaundice (1) and is frequently detected early compared with cancers arising elsewhere in the biliary tract. However, DBDC is associated with a poorer prognosis than intrahepatic BDC (1). Despite a surgical resection rate of ≥90.0% (1, 2), the 5-year survival rate for DBDC has been reported to be 25.0-55.0% (1-3). Furthermore, incidences of 5-year postoperative recurrences have been detected, which further reduce the true cure rate (3).

In the recent years, the biological malignancy of BDC has been elucidated through clinicopathological investigations. Similar to pancreatic cancer, BDC is frequently accompanied by lymph node metastasis (LNM) (3-5) and neural invasion (NI) (4, 6), both of which are factors affecting the prognosis, along with the surgical margin, tumour differentiation (1-3), invasion depth (4, 7), and curability (1, 2). In DBDC, the hepatic margin (2) and radial margin (2, 7) are associated with the prognosis. Radial margin evaluation is characteristic of the surgical margin and is important in DBDC (2, 7).

The phenomenon of tumour budding or 'sprouting' is histologically defined as a cancer cell cluster that has an undifferentiated phenotype comprising a single tumour cell or a cell cluster consisting of four tumour cells or lesser, with stromal invasive properties in the region of tumour development and advancement, and has been suggested to represent the initial phase of alterations for tumour invasion (8, 9). In some human cancers, budding has been reported to be strongly associated with epithelial-mesenchymal transition (EMT) (10-12), a phenomenon in which epithelial cells undergo morphological changes and become mesenchymal-like cells. This modification plays a significant role in cancer cell infiltration and metastasis. Budding and EMT are regulated by EMT-induced transcription factors. In the recent years, tumour budding has been linked to

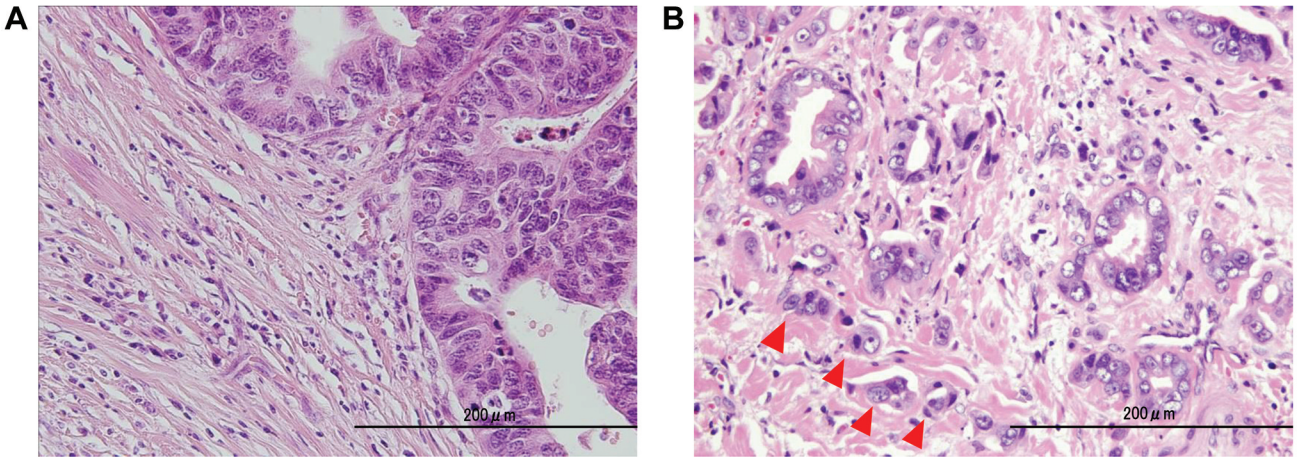


Figure 1. *Hematoxylin and eosin-stained sample slides from (A) the 'low' budding group and (B) the 'high' budding group. (×200).*

metastasis and prognosis in colon, gastric, oesophageal, laryngeal, skin, gallbladder, extrahepatic, ampullary, lung, and cutaneous squamous cell carcinomas (13-26). Tumour budding has attracted particular attention with respect to colon cancer and is important in determining treatment strategies in the early stages of the disease (27). In the biliary tract, tumour budding has been reported to correlate with survival in patients with T2 gallbladder cancer (22). Moreover, in ampullary cancer, tumour budding has been reported to be a more significant prognostic factor than other factors, such as the invasion depth and LNM (24). In extrahepatic cholangiocarcinoma, tumour budding has been reported to correlate with invasive clinicopathological features (23). Thus, the significance of tumour budding in the biliary tract has been steadily uncovered.

In this study, we performed a clinicopathological investigation of surgically resected cases to evaluate the significance of tumour budding in DBDC.

Patients and Methods

Patients. We retrospectively analysed 65 of 77 patients with DBDC who underwent pancreaticoduodenectomy at the Department of Surgery of Kurume University (Kurume, Japan) between 1995 and 2011. Twelve patients with papillary tumours were excluded. The study protocol was approved by the Institutional Review Board (No. 14096). All the participants provided written informed consent. This research was conducted in accordance with the 1964 Declaration of Helsinki and its later amendments.

Evaluation of tumour budding. The specimens removed during surgery were fixed in 10.0% neutral buffered formalin solution. Whole tissue sections (5.0 mm thick) were prepared for haematoxylin and eosin staining. The tissue sections were examined microscopically and tumour budding was evaluated histologically. Our evaluation reference was the study published by the International Tumour Budding Consensus Conference (ITBCC) 2016 (9). After selecting the area with the highest budding activity at the margins of the tumour, we counted the number of instances of budding at 200× magnification by applying a method known as the 'hot spot method' (9). Moreover, 0-4 cell clusters were designated as BD1, 5-9 cell clusters as BD2, and ≥10 cell clusters as BD3. Of these, BD1 were classified as the 'low' budding group (L group; Figure 1A) and BD2 and BD3 were classified as the 'high' budding group (H group; Figure 1B). The existing pathological parameter, 'poorly differentiated' was assumed to indicate five or more clusters. For all 65 cases, a pathologist conducted a parallel evaluation of budding, and in cases where budding was difficult to determine (16%), a third pathologist joined the evaluation and a final decision was reached.

Immunohistochemistry. As tumour budding is suggested to be associated with EMT, we studied the expression of vimentin, E-cadherin, and zinc finger E-box binding homeobox 1 (ZEB 1), an EMT-induced transcription factor, in regions of high budding activity (hotspots). We used the fully automated Bond-III system (Leica Microsystems) and onboard heat-induced antigen retrieval with epitope retrieval solution 1 for 10 min at 99˚C for vimentin and epitope retrieval solution 2 for 30 min at 99˚C for E-cadherin and ZEB1. Five-millimetre-thick sections of formalin-fixed, paraffin-embedded samples were mounted on glass slides and incubated for 30 min at room temperature with anti-rabbit monoclonal antibodies against vimentin (clone V9, dilution 1:9, DAKO, Santa Clara, CA, USA), E-cadherin (clone NCH-384, dilution 1:100, DAKO), and ZEB1 (clone 6935, dilution 1:200, Bethyl Laboratories, Montgomely, TX, USA). Subsequently, we incubated the slides for 30 min at room temperature with the refined polymer detection kit with horseradish peroxidase-polymer secondary antibody in the Band III automated system, and used 3.3'-Diaminobenzidine (DAB) as a substrate. To quantify ZEB1 staining, we followed the protocol described by Bronsert *et al*. (28). Granular stains in less than 1% of tumour cell nuclei were considered as negative, whereas smears stained with 1% or more in the presence of budding were considered positive.

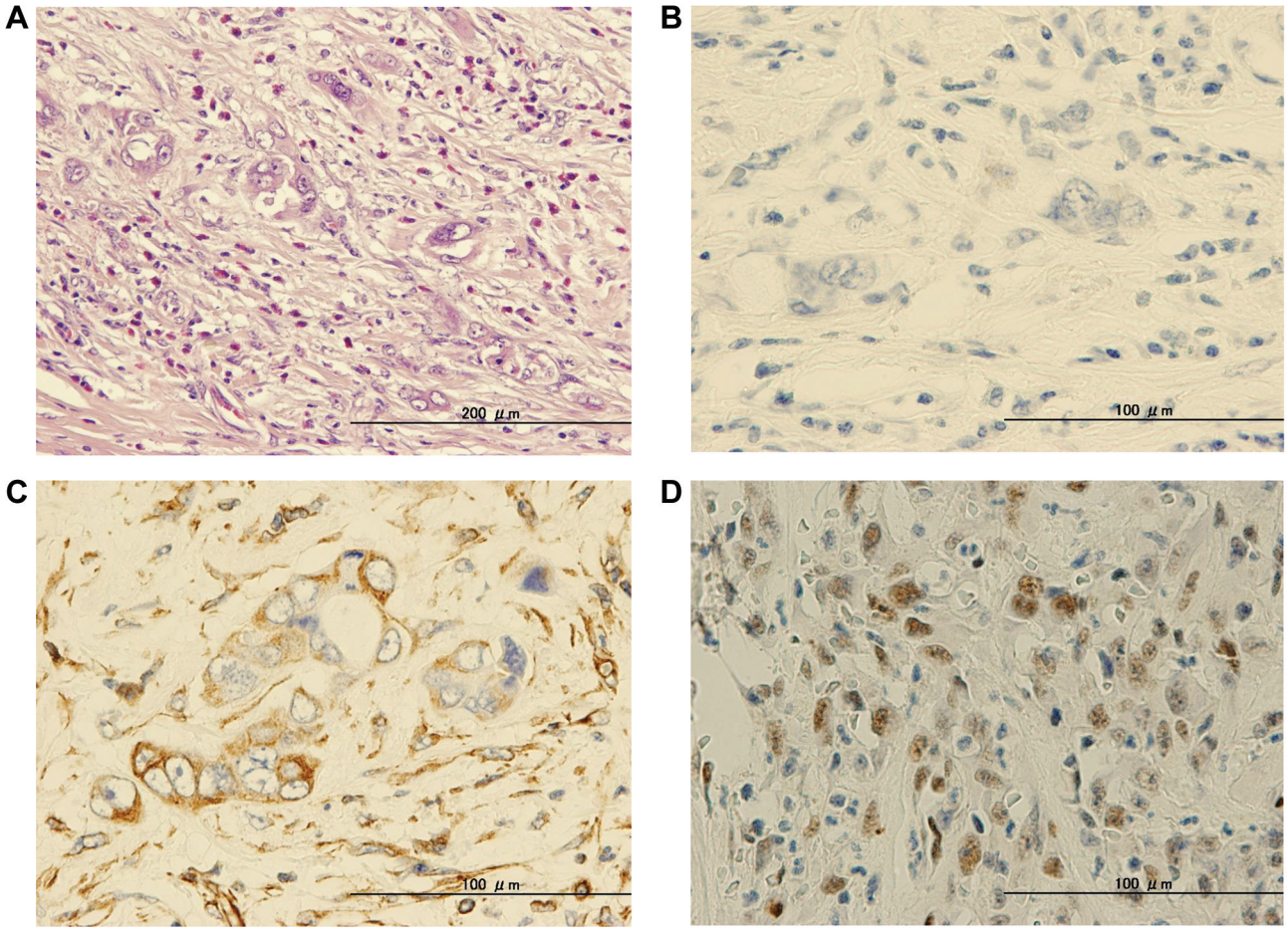


Figure 2. *A sample slide from (A) the 'high' budding group. (B) No signals for E-cadherin were detected. (C) Expression of vimentin was positive. (D) ZEB1 expression was observed in varying degrees in group H. It was consistent with the corresponding observation in the nuclei of the cells that exhibited budding. (×400).*

Pathological staging. The clinicopathological characteristics of each case were evaluated in accordance with the International Union Against Cancer (UICC) TNM Classification of Malignant Tumours (8th edition) (29). The hepatic ductal margin (HDM) and radial margin (RM) were designated in accordance with the general rules for clinical and pathological studies on cancer of the biliary tract (7th edition) (30). The RM did not include the cut end of the bile duct on the duodenal or hepatic side, signifying that the cut end was perpendicular to the bile duct.

Statistical analyses. The clinicopathological characteristics were compared between the groups using a chi-square test. The influence of each factor on the overall survival (OS) was tested in a univariate analysis using a Cox proportional hazards model. The significant factors were subjected to a multivariate analysis. The OS curves were calculated for each group using the Kaplan-Meier method and compared using a log-rank test. Statistical analyses were conducted using the JMP® Pro 11 software for Windows (SAS Institute, Cary, NC, USA). Statistical significance was set at $p<0.05$.

Results

Clinicopathological characteristics. The UICC definition (29) of T4 disease is 'a tumour involving the celiac axis, superior mesenteric artery, common hepatic artery, or portal vein'. Since cases of arterial invasion are not subjected to surgery at our institution, only two cases of T4 disease were observed (*i.e.*, portal vein invasion cases). DBDC patients (n=65) were classified into the L (n=12) and H (n=53) groups according to the extent of tumour budding. A photomicrograph of a case evaluated as high-budding is shown in Figure 2A. Individual cancer cells invade the interstitium or form vesicular stroma at the tumour margins. Immunostaining revealed negative E-cadherin expression (Figure 2B), and a positive signal for vimentin (Figure 2C). The expression of vimentin varied depending on the degree of budding; however, the expression of E-cadherin was clearly lower than that in highly

Table I. *Comparison of the clinicopathological characteristics of patients in the "high" (H; n=53) vs. "low" (L; n=12) budding groups.*

Characteristic	H group (n=53)	L group (n=12)	*p*-Value
Age, mean (y)	65.9±1.2	70.8±2.3	0.14
Sex, n (%)			
Male	39 (73.6)	8 (66.7)	
Female	14 (26.4)	4 (33.3)	0.63
Tumour size, mean (mm)	25.0±1.6	22.2±3.2	0.41
T-stage, n (%)			
1	0 (0.0)	1 (8.3)	
2	13 (24.5)	8 (66.7)	
3	38 (71.7)	3 (25.0)	
4	2 (3.8)	0 (0.0)	0.0051*
N-stage, n (%)			
0	19 (35.8)	10 (83.3)	
1	28 (52.8)	2 (16.7)	
2	6 (11.3)	0 (0.0)	0.0063*
Lymphatic invasion, n (%)			
Positive	49 (92.5)	9 (75.0)	
Negative	4 (7.5)	3 (25.0)	0.0782
Venous invasion, n (%)			
Positive	45 (84.9)	6 (50.0)	
Negative	8 (15.1)	6 (50.0)	0.0079*
Perineural invasion, n (%)			
Positive	50 (94.3)	9 (75.0)	
Negative	3 (5.7)	3 (25.0)	0.0366*
RM, n (%)			
Positive	5 (9.4)	0 (0.0)	
Negative	48 (90.6)	12 (100.0)	0.268
HDM, n (%)			
Positive	8 (15.1)	1 (8.3)	
Negative	45 (84.9)	11 (91.6)	0.540
Differentiation†, n (%)			
Well	17 (32.1)	8 (66.7)	
Moderate	24 (45.3)	4 (33.3)	
Poor	12 (22.6)	0 (0.0)	0.0477*
ZEB1, n (%)			<0.0001*
Positive	40 (75.5)	0 (0.0)	
Negative	13 (24.5)	12 (100.0)	
Stage, n (%)			
I	0	1 (8.3)	
IIA	19 (35.9)	9 (75.0)	
IIB	27 (50.9)	2 (16.7)	
IIIA	5 (9.43)	0	
IIIB	2 (3.77)	0	
IV	0	0	0.0169*

*$p<0.05$. †Differentiation of the adenocarcinoma. H: High; HDM: hepatic ductal margin; L: low; RM: radial margin.

differentiated sites in the same tumour. Although ZEB1 expression was evaluated immunohistochemically, it varied in group H samples. ZEB1 expression was observed in the nuclei of the cells that exhibited budding (Figure 2D).

We did not observe any significant differences in budding between the L and H groups of patients in relation to their clinicopathological characteristics, that is, age, sex, maximum tumour diameter, lymphatic invasion, RM, and HDM. In contrast, the invasion depth, LNM, venous/NI, tumour differentiation, disease stage, and ZEB1 expression were factors causing significant differences in budding between the two groups. The H group showed more marked signs in terms of the invasion depth ($p<0.01$), LNM ($p<0.01$), venous invasion ($p<0.01$), NI ($p<0.05$), and ZEB1 ($p<0.001$) than the L group. ZEB1 expression was not observed in the L group. The H group was also associated with a significantly higher proportion of patients with poor tumour differentiation ($p<0.05$). There were no cases of poorly differentiated tubular adenocarcinomas in the L group. The disease stage was also more advanced in the H group than in the L group ($p<0.05$; Table I).

Prognostic factors. Subsequent univariate analyses of potential prognostic factors for DBDC identified tumour budding, LNM, RM, ZEB1, and disease stage as statistically significant ($p<0.05$). These factors were then used in the multivariate analysis, and tumour budding and disease stage were found to be independent prognostic factors ($p<0.05$; Table II).

Survival analysis. The OS rates were estimated using the Kaplan-Meier method. A subsequent comparison of the OS rate between the H and L groups revealed that patients in the H group had a significantly poorer prognosis than patients in the L group ($p<0.0022$; Figure 3). The survival rate between the two groups was also examined with respect to the disease stage. There was only one case of stage I in the L group, and all 7 cases of stage III were H group cases. Therefore, we focused on stage II cases and evaluated them. In stage II, the prognosis in group H was significantly worse than that in group L ($p<0.0051$; Figure 4).

Discussion

Despite a high rate of surgical resection, DBDC is associated with poor prognosis (1-3). In recent years, the biological malignancy of DBDC has been elucidated through clinicopathological investigations. In the present study, we focused on the histological phenomenon of tumour budding that has been reported in various types of cancer, and investigated cases of surgically resected DBDC. We adopted the 'hot spot method' to evaluate tumour budding. Generally, the methods for evaluating tumour budding can be roughly divided into two categories. Method [1] is known as the 'hot spot method' in which the most advanced budding site is selected. Method [2] is the '5 high power field method' or the '10 high power field method', which evaluates the number of

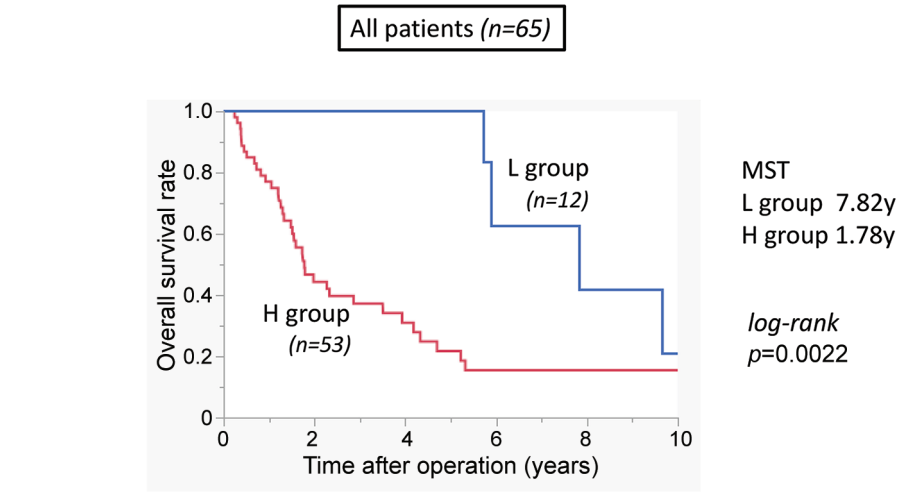
Table II. *Univariate and multivariate analyses of prognostic factors for survival in patients with distal bile duct cancer (n=65).*

Variable	Patients, n (%)	Univariate analysis		Multivariate analysis	
		HR (95%CI)	*p*-Value	HR (95%CI)	*p*-Value
Sex					
Male	47 (72.3)	1			
Female	18 (27.7)	0.90 (0.45-1.80)	0.7578		
BD					
L group†	12 (18.5)	1		1	
H group‡	53 (81.5)	4.48 (1.58-12.7)	0.0047*	3.91 (1.15-13.3)	0.0282*
T-stage					
1, 2	22 (33.8)	1			
3, 4	43 (66.2)	1.86 (0.94-3.69)	0.0741		
N-stage					
0	29 (49.6)	1		1	
1, 2	36 (55.4)	2.26 (1.20-4.48)	0.0139*	1.34 (0.61-2.90)	0.4645
Lymphatic invasion, n (%)					
Positive	58 (89.2)	2.66 (0.80-16.50)	0.1228		
Negative	7 (10.8)	1			
Venous invasion, n (%)					
Positive	51 (78.5)	1.62 (0.73-4.31)	0.2462		
Negative	14 (21.5)	1			
Perineural invasion, n (%)					
Positive	59 (90.8)	2.94 (0.90-18.1)	0.0800		
Negative	6 (9.2)	1			
RM					
Positive	5 (7.7)	5.50 (1.95-15.48)	0.0012*	1.71 (0.46-6.35)	0.465
Negative	60 (92.3)	1		1	
HDM					
Positive	9 (13.8)	1.85 (0.79-3.87)	0.1494		
Negative	56 (86.2)	1			
Differentiation¶					
Well	25 (38.5)	1			
Moderate, poor	40 (61.5)	1.67 (0.86-3.42)	0.1325		
ZEB1					
Positive	40 (61.5)	2.10 (1.09-4.10)	0.0264*	1.14 (0.49-2.64)	0.7541
Negative	25 (38.5)	1		1	
Stage					
I, II	58 (89.2)	1		1	
III	7 (10.8)	10.64 (3.71-30.5)	<0.0001*	6.11 (1.668-22.37)	0.0063*

*$p<0.05$. †"Low" budding group. ‡"High" budding group. ¶Differentiation of the adenocarcinoma. BD: Budding; CI: confidence interval; HDM: hepatic ductal margin; HR: hazard ratio; RM: radial margin.

instances of budding in several areas of the sample. With this method, there is a risk that the biological malignancy of budding will be underestimated because it is an evaluation based on the average value. We opted for the former method to avoid this outcome. In addition, several other studies have also used this evaluation method (12, 22, 24), and the 'hot spot method' was strongly recommended at ITBCC 2016 (9). We separated the patients into the L and H groups according to the extent of tumour budding and compared the clinicopathological factors between the two groups.

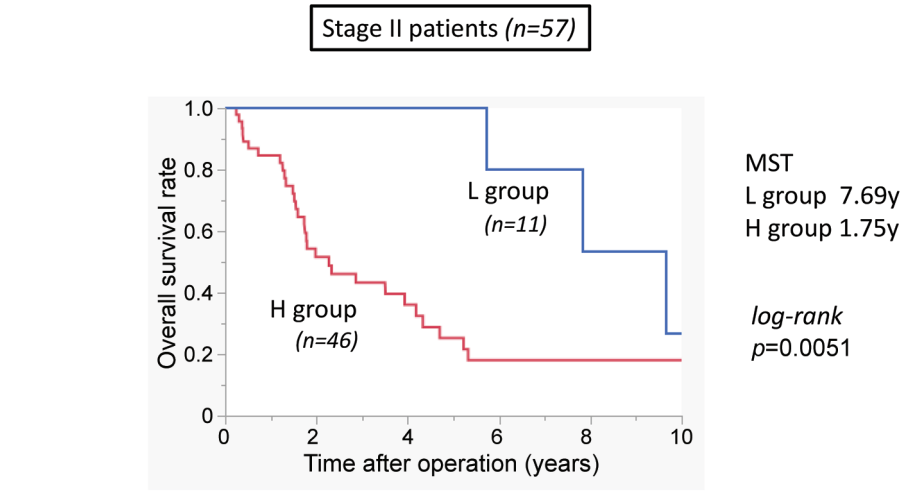
Compared with the L group, the H group was more advanced in terms of the depth of invasion, LNM, venous/NI, tumour differentiation, ZEB1, and disease stage. In oesophageal cancer patients, the tumour diameter, invasion depth, LNM, lymphatic/venous/NI, and tumour differentiation have been reported to be significantly associated with tumour budding (19, 20). In pulmonary adenocarcinoma patients, LNM, lymphatic/venous invasion, pleural infiltration, and disease stage have been reported to be significantly associated with tumour budding (21). Moreover, in patients with gallbladder cancer, invasion depth, LNM, and ampullary cancer patients, LNM, lymphatic/NI, tumour differentiation, and disease stage have also been reported to be significantly associated with tumour budding (22, 24). Therefore, it is conceivable that tumour budding is indicative of higher biological malignancy.



number at risk

L group	12	12	8	4	3	2
H group	53	20	11	6	4	4

Figure 3. *Kaplan-Meier curve of overall survival for patients with resected distal bile duct cancer (n=65) in the "low" and "high" budding groups.*



number at risk

L group	11	11	10	4	3	2
H group	46	20	11	6	4	4

Figure 4. *Kaplan-Meier curve of overall survival for stage II patients with resected distal bile duct cancer in the "low" and "high" budding groups.*

Moreover, there is a high probability that the histological phenomenon of tumour budding in patients with DBDC is responsible for the development of the tumour and its invasion and/or metastasis to other organs and tissues.

We subsequently analysed the pathological characteristics related to the prognosis of patients with DBDC. Univariate analysis identified tumour budding, LNM, RM, ZEB1, and the disease stage as significant prognostic factors between the two groups. A multivariate analysis verified that tumour budding and disease stage were independent prognostic factors. Tumour budding has been reported to be significantly associated with OS in various cancer types. It

has been reported that tumour budding is an independent prognostic factor for Stage II colon cancer (specifically T3N0M0 patients) (13, 14). Ueno *et al*. concluded that tumour budding (<5 instances are visible per high-power field of view) is a condition for follow-up treatments after endoscopic therapy for early-stage colon cancer (27). In pulmonary adenocarcinoma patients with a tumour diameter ≤3.0 cm, tumour budding has been identified as a prognostic factor, along with LNM, lymphatic/venous invasion, and pleural infiltration (21). Tumour budding has also been reported to be significantly associated with the prognosis in oesophageal cancer patients (19, 20). Specifically, Brown *et al*. (19) concluded that tumour budding is an independent prognostic factor in addition to age (>65 years), LNM, and disease stage. For gastric cancer patients, the L group had a significantly longer OS time than the H group (18). Moreover, it has been reported that in the biliary tract, tumour budding, invasion depth, LNM, and cut end factors are significant prognostic factors for ampullary cancer, and tumour budding was also reported to be a stronger prognostic factor than invasion depth, LNM (24). Ogino *et al*. reported that budding is an independent adverse prognostic factor for extrahepatic cholangiocarcinoma (23). In view of this and similar reports in other organs, tumour budding in DBDC is considered to be closely correlated with prognosis.

In an analysis of survival involving all 65 patients in our study, the H group was associated with a significantly poorer prognosis than the L group. However, due to the differences in clinicopathological backgrounds between the two groups, a potential for factors other than tumour budding to influence survival was observed. Therefore, we compared the OS rates between the H and L groups in relation to the disease stage. A significant difference in the prognosis was observed in stage II cases. Similar trends have been observed in other types of cancer. Tumour budding has been reported to be significantly associated with survival in American Joint Committee on Cancer/UICC stage II colon cancer (13, 14), T1 oesophageal cancer (20), and pulmonary adenocarcinoma with a tumour diameter ≤3.0 cm (25). In a study of the invasion depth (T2/T3) in patients with gallbladder cancer, tumour budding was reported to be significantly associated with the survival in T2 cases (22). In view of this, it is highly likely that tumour budding is more significant in early-stage cancer.

In recent years, epithelial-mesenchymal transition has been shown to be involved in the progression of epithelial cancer. It has been noted that tumour budding could mirror the morphological changes in epithelial-mesenchymal transition (10, 11, 16, 23). Tumour budding has also been reported to be involved in the loss of E-cadherin, a tumour adhesion factor that activates the WNT signalling pathway and the breakdown of the extracellular matrix (10). E-cadherin expression was not detected at the budding sites. ZEB1, another factor involved in EMT, was observed in the budding sites to varying degrees, which is in line with previous studies (28, 31). The known EMT-induced transcription factors Snail, Slug, ZEB1, and ZEB2 directly inhibit E-cadherin and Twist, which indirectly regulate the E-cadherin expression. ZEB1 is known to promote invasion/metastasis in human cancer cells and has been observed in the pancreatic, breast, urothelial, uterine body, and colon cancers (28, 32-34). In our study, ZEB1 expression was higher in the H group than in the L group during budding, a morphological change caused by EMT. Hence, ZEB1 may be involved in the budding phenomenon observed in patients with bile duct cancer. Although the expression of ZEB1 was predicted to be associated with the prognosis, based on our univariate analysis, it was not an independent prognostic factor. This may be because ZEB1 expression is low in immunohistochemical staining, making its detection difficult. Moreover, we only examined sections with high budding activity; therefore, EMT changes may not necessarily be observed in these areas. There is also the possibility that ZEB1 may be involved in the budding phenomenon in DBDC cases, requiring further studies on the mechanism of budding.

The UICC stage is the main predictor of prognosis in DBDC. In our study, we also reported that the prognosis worsened as the stage progressed, although we were not able to detect any statistically significant differences. However, the concept of disease stage did not consider the phenomenon of tumour budding, which was the focus of this study.

The phenomenon of tumour budding in DBDC is frequently accompanied by LNM, vascular/NI, *etc*., and is therefore considered to have a high potential for biological malignancy. Incorporating the stage in our analysis showed that in Stage II, the prognosis was significantly worse in group H than in group L. Consequently, patients with tumour budding have a poorer prognosis even if the disease stage is relatively early and the biological malignancy is considered high. In addition, we believe that it is difficult to ascertain whether budding occurred preoperatively.

As mentioned above, recurrence frequently occurs in cases of bile duct cancer, even when curative resection is performed. In this study, 41 of the 65 patients died. As most postsurgical follow-up sessions were performed at other facilities, it was not possible to determine the nature of recurrence and cause of death in all cases. The cause of death was confirmed in only 11 cases: six cases of recurrent liver metastasis, three cases of recurrent lung metastasis, two cases of local recurrence, and one case of recurrent peritoneal dissemination (overlapping). Although it is interesting to consider whether there is a difference in the type of recurrence or metastasis depending on the degree of budding, such an analysis was not feasible in our study due to the lack of documentation. We hope to address this topic in greater detail in the future.

In conclusion, we clinically examined the significance of tumour budding in DBDC cases in this study. During this evaluation, it was possible to select cases with a poor prognosis that could not be distinguished by stage alone. Besides, the budding phenomenon can easily be determined by HE staining, the fact that this method can be implemented relatively easily and inexpensively in any facility is also a notable advantage. The budding phenomenon was an independent prognostic factor in DBDC cases, and our findings suggest a role for ZEB1 in this phenomenon. Characterization of the mechanism of tumour budding can lead to a better understanding of the pathology of distal bile duct cancer and its poor prognosis, which may lead to the development of more effective treatments for distal bile duct cancer.

Conflicts of Interest

The Authors have no conflicts of interest to declare in relation to this study.

Authors' Contributions

Goichi Nakayama designed the study and prepared the first draft of the manuscript. Goichi Nakayama and Toru Hisaka contributed to the analysis and interpretation of the data and assisted with the preparation of the manuscript. Goichi Nakayama, Toru Hisaka, Yoshinori Naito, Jun Akiba, and Hirohisa Yano performed pathological analyses and evaluations. All other Authors contributed to the collection and interpretation of data and critically reviewed the manuscript. All the Authors approved the final version of the manuscript and ensured that the questions related to the accuracy or completeness of the working part were properly investigated and resolved.

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References

1 DeOliveira ML, Cunningham SC, Cameron JL, Kamangar F, Winter JM, Lillemoe KD, Choti MA, Yeo CJ and Schulick RD: Cholangiocarcinoma: thirty-one-year experience with 564 patients at a single institution. Ann Surg *245(5)*: 755-762, 2007. PMID: 17457168. DOI: 10.1097/01.sla.0000251366.62632.d3

2 Todoroki T, Kawamoto T, Koike N, Fukao K, Shoda J and Takahashi H: Treatment strategy for patients with middle and lower third bile duct cancer. Br J Surg *88(3)*: 364-370, 2001. PMID: 11260100. DOI: 10.1046/j.1365-2168.2001.01685.x

3 Jang JY, Kim SW, Park DJ, Ahn YJ, Yoon YS, Choi MG, Suh KS, Lee KU and Park YH: Actual long-term outcome of extrahepatic bile duct cancer after surgical resection. Ann Surg *241(1)*: 77-84, 2005. PMID: 15621994. DOI: 10.1097/01.sla.0000150166.94732.88

4 Hong SM, Cho H, Lee OJ and Ro JY: The number of metastatic lymph nodes in extrahepatic bile duct carcinoma as a prognostic factor. Am J Surg Pathol *29(9)*: 1177-1183, 2005. PMID: 16096407. DOI: 10.1097/01.pas.0000160978.77833.d7

5 Kiriyama M, Ebata T, Aoba T, Kaneoka Y, Arai T, Shimizu Y, Nagino M and Nagoya Surgical Oncology Group: Prognostic impact of lymph node metastasis in distal cholangiocarcinoma. Br J Surg *102(4)*: 399-406, 2015. PMID: 25611179. DOI: 10.1002/bjs.9752

6 Sakamoto Y, Kosuge T, Shimada K, Sano T, Ojima H, Yamamoto J, Yamasaki S, Takayama T and Makuuchi M: Prognostic factors of surgical resection in middle and distal bile duct cancer: an analysis of 55 patients concerning the significance of ductal and radial margins. Surgery *137(4)*: 396-402, 2005. PMID: 15800484. DOI: 10.1016/j.surg.2004.10.008

7 Wakai T, Shirai Y, Moroda T, Yokoyama N and Hatakeyama K: Impact of ductal resection margin status on long-term survival in patients undergoing resection for extrahepatic cholangiocarcinoma. Cancer *103(6)*: 1210-1216, 2005. PMID: 15685618. DOI: 10.1002/cncr.20906

8 Morodomi T, Isomoto H, Shirouzu K, Kakegawa K, Irie K and Morimatsu M: An index for estimating the probability of lymph node metastasis in rectal cancers. Lymph node metastasis and the histopathology of actively invasive regions of cancer. Cancer *63(3)*: 539-543, 1989. PMID: 2912530. DOI: 10.1002/1097-0142(19890201)63:3<539::aid-cncr2820630323>3.0.co;2-s

9 Lugli A, Kirsch R, Ajioka Y, Bosman F, Cathomas G, Dawson H, El Zimaity H, Fléjou JF, Hansen TP, Hartmann A, Kakar S, Langner C, Nagtegaal I, Puppa G, Riddell R, Ristimäki A, Sheahan K, Smyrk T, Sugihara K, Terris B, Ueno H, Vieth M, Zlobec I and Quirke P: Recommendations for reporting tumor budding in colorectal cancer based on the International Tumor Budding Consensus Conference (ITBCC) 2016. Mod Pathol *30(9)*: 1299-1311, 2017. PMID: 28548122. DOI: 10.1038/modpathol.2017.46

10 Dawson H and Lugli A: Molecular and pathogenetic aspects of tumor budding in colorectal cancer. Front Med (Lausanne) *2*: 11, 2015. PMID: 25806371. DOI: 10.3389/fmed.2015.00011

11 Cao H, Xu E, Liu H, Wan L and Lai M: Epithelial-mesenchymal transition in colorectal cancer metastasis: A system review. Pathol Res Pract *211(8)*: 557-569, 2015. PMID: 26092594. DOI: 10.1016/j.prp.2015.05.010

12 Rogers AC, Winter DC, Heeney A, Gibbons D, Lugli A, Puppa G and Sheahan K: Systematic review and meta-analysis of the impact of tumour budding in colorectal cancer. Br J Cancer *115(7)*: 831-840, 2016. PMID: 27599041. DOI: 10.1038/bjc.2016.274

13 Petrelli F, Pezzica E, Cabiddu M, Coinu A, Borgonovo K, Ghilardi M, Lonati V, Corti D and Barni S: Tumour budding and survival in stage II colorectal cancer: a systematic review and pooled analysis. J Gastrointest Cancer *46(3)*: 212-218, 2015. PMID: 25994502. DOI: 10.1007/s12029-015-9716-1

14 Betge J, Kornprat P, Pollheimer MJ, Lindtner RA, Schlemmer A, Rehak P, Vieth M and Langner C: Tumor budding is an independent predictor of outcome in AJCC/UICC stage II colorectal cancer. Ann Surg Oncol *19(12)*: 3706-3712, 2012. PMID: 22669453. DOI: 10.1245/s10434-012-2426-z

15 Karlberg M, Stenstedt K, Hallström M, Ragnhammar P, Lenander C and Edler D: Tumor budding *versus* mismatch repair status in colorectal cancer - an exploratory analysis. Anticancer Res *38(8)*: 4713-4721, 2018. PMID: 30061240. DOI: 10.21873/anticanres.12778

16 Matsui H, Homma S, Hatanaka KC, Sawada A, Imaizumi K, Yoshida T, Ichikawa N, Emoto S, Miyaoka Y, Takahashi N, Matsuno Y, Hatanaka Y and Taketomi A: Novel prognostic stratification factor based on the pathological profile of the invasive front in colorectal cancer. Anticancer Res *41(7)*: 3429-3438, 2021. PMID: 34230138. DOI: 10.21873/anticanres.15130
17 Shirouzu K: Significance of tumor budding in early colorectal cancer based on micromorphologic and statistical studies. In: Muto T, Mochizuki H, Masaki T, editors. Tumor Budding in Colorectal Cancer: Recent Progress in Colorectal Cancer Research. New York, Nova Biomedical Books, pp. 85-103, 2006.
18 Gabbert HE, Meier S, Gerharz CD and Hommel G: [New histomorphologic prognostic parameters in stomach cancer. A uni- and multivariate analysis of 445 patients]. Chirurg *63(8)*: 647-655, 1992. PMID: 1395861.
19 Brown M, Sillah K, Griffiths EA, Swindell R, West CM, Page RD, Welch IM and Pritchard SA: Tumour budding and a low host inflammatory response are associated with a poor prognosis in oesophageal and gastro-oesophageal junction cancers. Histopathology *56(7)*: 893-899, 2010. PMID: 20636792. DOI: 10.1111/j.1365-2559.2010.03559.x
20 Teramoto H, Koike M, Tanaka C, Yamada S, Nakayama G, Fujii T, Sugimoto H, Fujiwara M, Suzuki Y and Kodera Y: Tumor budding as a useful prognostic marker in T1-stage squamous cell carcinoma of the esophagus. J Surg Oncol *108(1)*: 42-46, 2013. PMID: 23609421. DOI: 10.1002/jso.23341
21 Sarioglu S, Acara C, Akman FC, Dag N, Ecevit C, Ikiz AO, Cetinayak OH, Ada E and for Dokuz Eylül Head and Neck Tumour Group (DEHNTG): Tumor budding as a prognostic marker in laryngeal carcinoma. Pathol Res Pract *206(2)*: 88-92, 2010. PMID: 19959297. DOI: 10.1016/j.prp.2009.09.006
22 Kai K, Kohya N, Kitahara K, Masuda M, Miyoshi A, Ide T, Tokunaga O, Miyazaki K and Noshiro H: Tumor budding and dedifferentiation in gallbladder carcinoma: potential for the prognostic factors in T2 lesions. Virchows Arch *459(4)*: 449-456, 2011. PMID: 21785869. DOI: 10.1007/s00428-011-1131-9
23 Ogino M, Nakanishi Y, Mitsuhashi T, Hatanaka Y, Amano T, Marukawa K, Nitta T, Ueno T, Ono M, Kuwabara S, Yamada T and Hirano S: Impact of tumour budding grade in 310 patients who underwent surgical resection for extrahepatic cholangiocarcinoma. Histopathology *74(6)*: 861-872, 2019. PMID: 30667537. DOI: 10.1111/his.13827
24 Ohike N, Coban I, Kim GE, Basturk O, Tajiri T, Krasinskas A, Bandyopadhyay S, Morohoshi T, Shimada Y, Kooby DA, Staley CA, Goodman M and Adsay NV: Tumor budding as a strong prognostic indicator in invasive ampullary adenocarcinomas. Am J Surg Pathol *34(10)*: 1417-1424, 2010. PMID: 20871215. DOI: 10.1097/PAS.0b013e3181f0b05a
25 Yamaguchi Y, Ishii G, Kojima M, Yoh K, Otsuka H, Otaki Y, Aokage K, Yanagi S, Nagai K, Nishiwaki Y and Ochiai A: Histopathologic features of the tumor budding in adenocarcinoma of the lung: tumor budding as an index to predict the potential aggressiveness. J Thorac Oncol *5(9)*: 1361-1368, 2010. PMID: 20631633. DOI: 10.1097/JTO0b013e3181eaf2f3
26 Karayannopoulou G, Panteris E and Kanitakis J: Tumour budding is an independent predictive factor of cutaneous squamous-cell carcinoma aggressiveness. Anticancer Res *40(5)*: 2695-2699, 2020. PMID: 32366414. DOI: 10.21873/anticanres.14240
27 Ueno H, Mochizuki H, Hashiguchi Y, Shimazaki H, Aida S, Hase K, Matsukuma S, Kanai T, Kurihara H, Ozawa K, Yoshimura K and Bekku S: Risk factors for an adverse outcome in early invasive colorectal carcinoma. Gastroenterology *127(2)*: 385-394, 2004. PMID: 15300569. DOI: 10.1053/j.gastro.2004.04.022
28 Bronsert P, Kohler I, Timme S, Kiefer S, Werner M, Schilling O, Vashist Y, Makowiec F, Brabletz T, Hopt UT, Bausch D, Kulemann B, Keck T and Wellner UF: Prognostic significance of Zinc finger E-box binding homeobox 1 (ZEB1) expression in cancer cells and cancer-associated fibroblasts in pancreatic head cancer. Surgery *156(1)*: 97-108, 2014. PMID: 24929761. DOI: 10.1016/j.surg.2014.02.018
29 Brierley JD, Gospodarowicz MK and Wittekind C: TNM classification of malignant tumours, 8th edition. Wiley-Blackwell, 2016.
30 Japanese Society of Hepato-Biliary-Pancreatic Surgery: General rules for clinical and pathological studies on cancer of the biliary tract. 7th edition. Tokyo, Kanehara & Co., Ltd, 2021.
31 Nitta T, Mitsuhashi T, Hatanaka Y, Miyamoto M, Oba K, Tsuchikawa T, Suzuki Y, Hatanaka KC, Hirano S and Matsuno Y: Prognostic significance of epithelial-mesenchymal transition-related markers in extrahepatic cholangiocarcinoma: comprehensive immunohistochemical study using a tissue microarray. Br J Cancer *111(7)*: 1363-1372, 2014. PMID: 25077440. DOI: 10.1038/bjc.2014.415
32 Schulte J, Weidig M, Balzer P, Richter P, Franz M, Junker K, Gajda M, Friedrich K, Wunderlich H, Östman A, Petersen I and Berndt A: Expression of the E-cadherin repressors Snail, Slug and Zeb1 in urothelial carcinoma of the urinary bladder: relation to stromal fibroblast activation and invasive behaviour of carcinoma cells. Histochem Cell Biol *138(6)*: 847-860, 2012. PMID: 22820858. DOI: 10.1007/s00418-012-0998-0
33 Singh M, Spoelstra NS, Jean A, Howe E, Torkko KC, Clark HR, Darling DS, Shroyer KR, Horwitz KB, Broaddus RR and Richer JK: ZEB1 expression in type I *vs*. type II endometrial cancers: a marker of aggressive disease. Mod Pathol *21(7)*: 912-923, 2008. PMID: 18487993. DOI: 10.1038/modpathol.2008.82
34 Spaderna S, Schmalhofer O, Hlubek F, Berx G, Eger A, Merkel S, Jung A, Kirchner T and Brabletz T: A transient, EMT-linked loss of basement membranes indicates metastasis and poor survival in colorectal cancer. Gastroenterology *131(3)*: 830-840, 2006. PMID: 16952552. DOI: 10.1053/j.gastro.2006.06.016

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