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Prognostic significance of tumor budding in patients with pancreatic invasive ductal carcinoma who received neoadjuvant therapy

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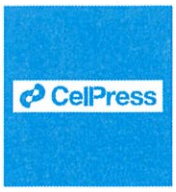
Heliyon 10 (2024) e23928



Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Prognostic significance of tumor budding in patients with pancreatic invasive ductal carcinoma who received neoadjuvant therapy

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ARTICLE INFO

Keywords:
Pancreas
Carcinoma
Tumor budding
Prognosis
Neoadjuvant therapy

ABSTRACT

Neoadjuvant therapy is commonly used for invasive pancreatic ductal carcinoma (PDAC). Tumor budding and high podoplanin expression in cancer-associated fibroblasts (CAFs) are prognostic factors in patients with various carcinomas including PDAC who have not received neoadjuvant therapy. In this study, we investigated whether tumor budding and podoplanin-positive CAFs are associated with outcomes in Japanese PDAC patients with neoadjuvant therapy. Histopathological findings of surgically resected PDACs with neoadjuvant therapy from 2005 to 2018 were reviewed (n = 97). With reference to International Tumor Budding Consensus Conference recommendations, tumors were evaluated for budding at 20 × magnification (/0.785 mm^2) and at 40 × magnification (/0.237 mm^2; mean number of fields: 3) for podoplanin expression in CAFs (%). Overall survival, disease-free survival, and disease-specific survival (DSS) were analyzed using the log-rank test and Cox proportional hazards model. After adjusting for T category, N category, resection margin, and adjuvant therapy, multivariate analyses demonstrated that tumor budding at 40 × magnification was an independent prognostic factor for worse DSS (hazard ratio: 2.41, p = 0.022). Tumor budding at 20 × magnification and podoplanin-positive CAFs tended to be associated with worse DSS; however, these findings were not statistically significant. Our findings indicate that tumor budding is an independent prognostic factor in PDAC patients with neoadjuvant therapy.

1. Introduction

The annual incidence of pancreatic carcinoma is increasing worldwide, and the 5-year relative survival rate for pancreatic carcinoma is 11 %, the lowest among all cancers in the United States [1]. Although resection is the most effective means of achieving long-term survival [2,3], it enables a median overall survival (OS) of only 11–23 months, with a 5-year OS rate of approximately 20 % [4]. In Japan, pancreatic carcinoma is the fourth and third leading cause of cancer-related death in men and women, respectively [5]. It

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https://doi.org/10.1016/j.heliyon.2023.e23928
Received 9 July 2023; Received in revised form 13 December 2023; Accepted 15 December 2023
Available online 19 December 2023

Abbreviation list

CAFs	cancer-associated fibroblasts
NAC	neoadjuvant chemotherapy
CK	cytokeratin
DFS	disease-free survival
DSS	disease-specific survival
H&E	hematoxylin and eosin
HPF	high-power field
ITBCC	International Tumor Budding Consensus Conference
OS	overall survival
PDAC	pancreatic ductal carcinoma
EMT	epithelial–mesenchymal transition

has become clear that even for resectable carcinomas, better outcomes can be obtained by administering neoadjuvant chemotherapy before performing surgery [4]. Therefore, in recent years, we have made most pathological diagnoses of pancreatic carcinomas on surgical specimens obtained by resection after neoadjuvant therapy. Identifying prognostic factors in patients with pancreatic carcinoma who have received neoadjuvant therapy has assumed greater importance. Several prognostic factors for patients with PDAC who have received neoadjuvant therapy have been reported [4,6], but analysis of histological prognostic factors is not sufficient.

Histomorphological patterns such as lymphovascular and perineural invasion are known to be significant prognostic factors in various tumors, including colorectal, prostate, breast, and pancreatic adenocarcinomas [7–10]. Additionally, tumor budding, which is defined as a single tumor cell or a cluster of up to four tumor cells in the stroma at the outer edge of the tumor [11], has also been recognized as a prognostic factor in the carcinomas of various organs [12–14]. In particular, a scoring system was established at the International Tumor Budding Consensus Conference (ITBCC) for colorectal carcinoma [11]. Tumor budding is also known to be an important prognostic factor in pancreatic carcinoma [15–18]; however, its association with neoadjuvant therapy has not yet been reported.

In addition, interactions between carcinoma cells and surrounding cancer-associated fibroblasts (CAFs) play an important role in carcinoma progression. It is known that CAFs enhance the ability of carcinoma cells to proliferate and infiltrate through excessive secretion of various extracellular matrix proteins and activation of extracellular matrix proteinases. Additionally, it has been reported that the production of inflammatory cytokines and angiogenesis factors induces mobilization and angiogenesis of other carcinoma stromal cells such as tumor-associated macrophages, promoting the proliferation, infiltration, and metastasis of carcinoma cells [19,20]. Podoplanin expression in CAFs has been reported to predict poor prognosis in various cancer types, including pancreatic carcinoma [21]. Podoplanin is a mucoprotein with a molecular weight of 38 kDa that is known as a lymphatic endothelial cell marker and is useful for distinguishing between lymphatic vessels and blood vessels [22]. It is also a useful marker for tumor-related lymphangiogenesis [23,24]. Podoplanin staining is seen in tumors such as testicular germ cell tumors [25], Kaposi's sarcoma [26], and mesothelioma [27].

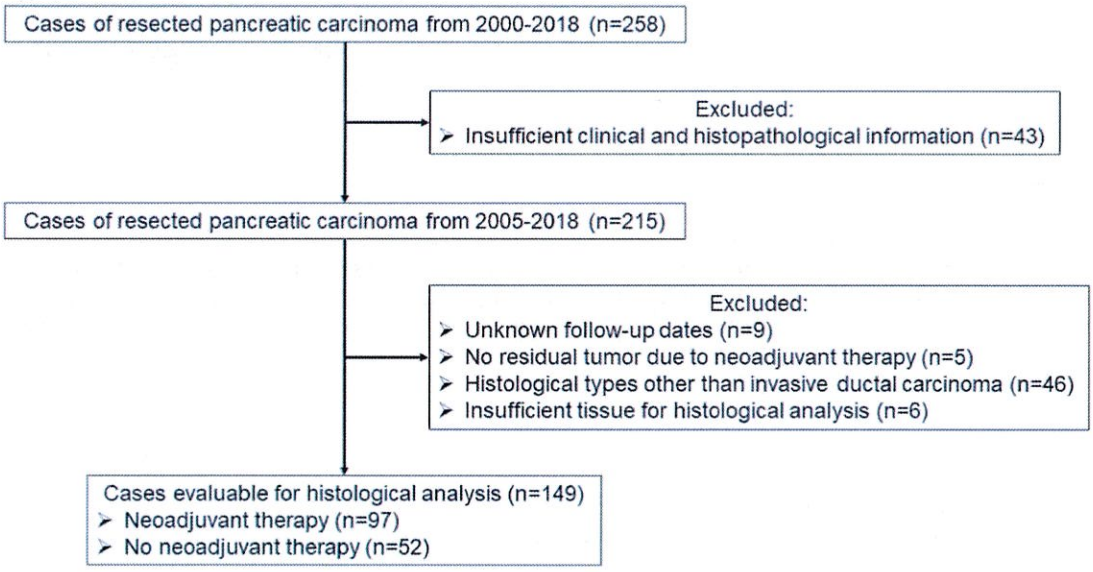

Fig. 1. Flowchart of histological analysis of the study cohort.

Recently, the relationship between tumor budding and podoplanin expression in tumor cells has been examined in oral squamous cell carcinoma [28,29]. In addition, studies in oral tongue squamous cell carcinoma and colorectal carcinoma have investigated the relationship between tumor budding and CAFs, along with other factors, and prognosis [30,31].

For both tumor budding and CAFs, patient groups receiving neoadjuvant treatment were excluded in most publications. In this study, we investigated the relationship between tumor budding and podoplanin expression in CAFs as well as prognosis in pancreatic carcinomas resected after neoadjuvant therapy by reviewing histopathological findings.

2. Materials and methods

2.1. Patients

This retrospective study was approved by the Institutional Review Board of Kagawa University (approval number: 2020-090). All methods were performed in accordance with the relevant guidelines and regulations. Informed consent was obtained from all patients included in this study.

We reviewed the records of 258 invasive PDAC patients who had undergone pancreatic resection at Kagawa University between 2000 and 2018. Cases from 2000 to 2004 were excluded because of insufficient clinical information and histopathological findings. Additionally, after excluding patients with unknown follow-up dates or no residual tumor after neoadjuvant therapy, and histological types other than invasive ductal carcinoma, 149 patients were included in the analysis (neoadjuvant therapy: $n = 97$; no neoadjuvant therapy: $n = 52$) (Fig. 1). In this study, hypofractionated external-beam radiation (30 Gy in 10 fractions) with concurrent S-1 (60 mg/m2/day) was administered 5 days per week for 2 weeks in 47 patients. Subsequently, standard external-beam radiation (50 Gy in 25 fractions) with concurrent S-1 (60 mg/m^2/day) was administered 5 days per week for 5 weeks in 36 patients from April 2016. In contrast, 10 patients with arterial invasion (BR-A) PDAC underwent neoadjuvant chemotherapy (NAC). The NAC regimen included a combination of gemcitabine and nanoparticle albumin-bound paclitaxel (nab-PTX) or a combination of fluorouracil, leucovorin,

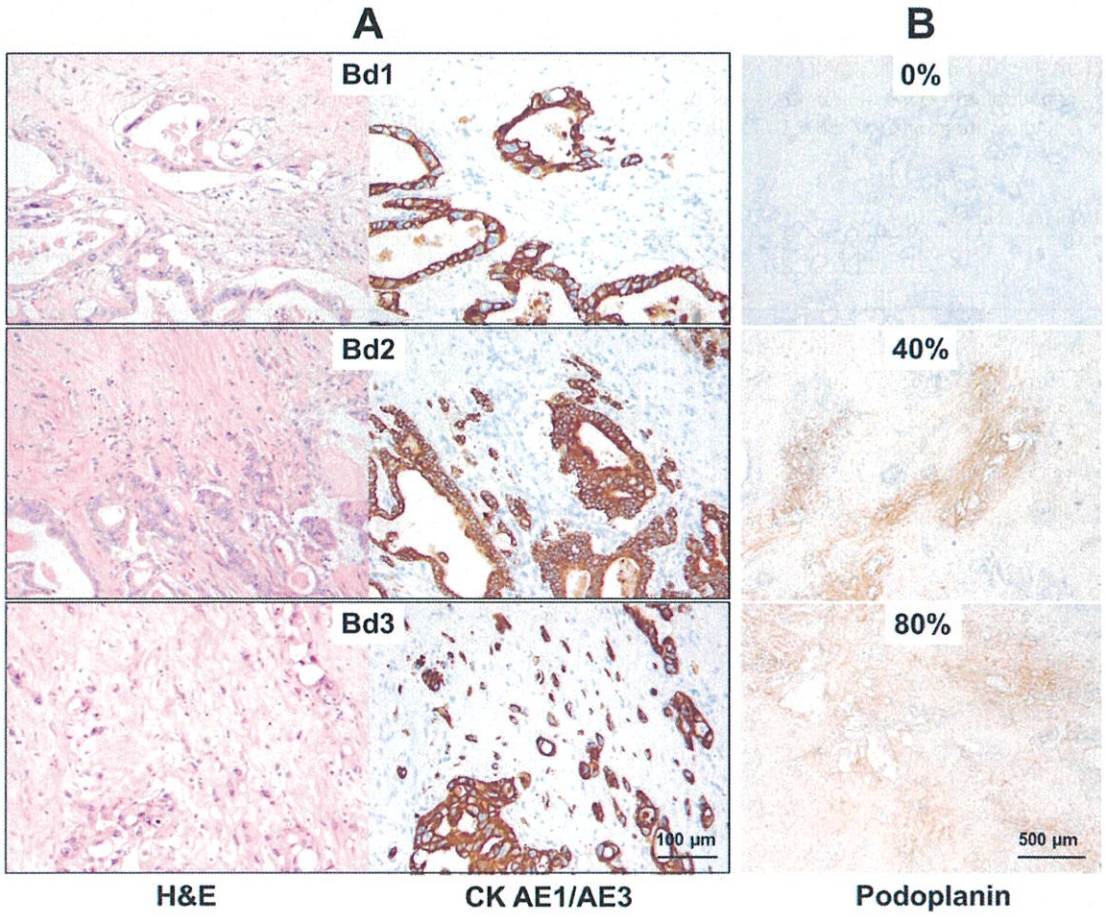

Fig. 2. Examples of tumor budding and podoplanin-positive cancer-associated fibroblasts. A: Invasive ductal carcinomas with low tumor budding (Bd 1: 0–4 buds), intermediate tumor budding (Bd 2: 5–9 buds), and high tumor budding (Bd 3: ≥10 buds) (original magnification: 200 ×). Left hand panels: hematoxylin and eosin staining; central panels: cytokeratin AE1/AE3 staining. B: Podoplanin-positive cancer-associated fibroblasts (original magnification: 40 ×).

irinotecan, and oxaliplatin (FOLFIRINOX) for 2 or 3 months as standard NAC. Additionally, four patients with BR-A underwent chemoradiotherapy after NAC. Clinical data were collected from a prospectively maintained pancreatic carcinoma database. Tumor-node-metastasis (TNM) stages were assigned on the basis of the eighth edition of the American Joint Committee on Cancer TNM Staging Manual [32].

2.2. Immunohistochemistry

We cut 4-μm sections from stored 10 % buffered formalin-fixed, paraffin-embedded tumor blocks, which were then stained with anti-cytokeratin (CK) AE1/AE3 antibody (clone AE1/AE3, ready to use; Dako, Carpinteria, CA, USA), a marker of epithelial tumor cells that highlights areas of tumor budding, and anti-podoplanin antibody (clone D2-40, ready to use; Nichirei Bioscience, Tsukiji, Tokyo, Japan), a marker of CAFs. We used diaminobenzidine as the chromogen and hematoxylin as the nuclear counterstain. Positive control tissues were stained in parallel with the study cases.

2.3. Histological evaluation

Using a Nikon Eclipse 55i microscope (Nikon, Konan, Tokyo, Japan) with a standard 22-mm-diameter eyepiece, tumor slides that had been stained with CK AE1/AE3 and podoplanin were reviewed by a pathologist (I.E.) who was blinded to patient outcomes. Tumor budding was defined as a single tumor cell or a cluster of up to four tumor cells [11]. The median number of tumor slides per case was five. After examining all tumor slides at 4 × magnification, one representative slide with well-retained tumor cells containing the margin of the tumor was selected and tumor budding hotspots at the invasive front were identified at 10 × magnification. For cases with few cancer cells remaining after treatment, the block with the most cancer cells remaining was selected. For cases in which it was difficult to identify the invasive front on the slides, we also referred to macroscopic images. At these hotspots, tumor buds were counted in accordance with the following two methods: (1) with reference to ITBCC recommendations, tumor budding was assessed in a field measuring 0.785 mm^2 in one hotspot at 20 × magnification [11]; and (2) three representative fields of 0.237 mm^2 within hotspots were selected randomly, and the number of tumor buds in each was counted at 40 × magnification [17]. The mean number of tumor buds over the three fields was then calculated. We used the following three-tier system to categorize bud counts: Bd 1 (0–4 buds), Bd 2 (5–9 buds), and Bd 3 (≥10 buds) (Fig. 2A) [11].

Scoring of podoplanin expression in CAFs was made on the basis of the distribution (%) of staining in one low-power field within a hotspot at 4 × magnification (Fig. 2B). To facilitate evaluation, lymphatic endothelial cells served as an internal positive control.

Table 1
Clinicopathological characteristics.

Variables	All patients		No neoadjuvant therapy		Neoadjuvant therapy	
	N	%	N	%	N	%
Age, years						
<70	69	46	24	46	45	46
≥70	80	54	28	54	52	54
Sex						
Female	64	43	19	37	45	46
Male	85	57	33	63	52	54
Tumor location						
Head/neck	89	60	28	54	61	63
Body/tail	60	40	24	46	36	37
T category						
T1	40	27	8	15	32	33
T2	84	56	32	62	52	54
T3	25	17	12	23	13	13
N category						
N0	83	56	28	54	55	57
N1	52	35	17	33	35	36
N2	14	9	7	13	7	7
Histologic grade						
G1	70	47	25	48	45	46
G2	58	39	21	40	37	38
G3	21	14	6	12	15	15
Neoadjuvant						
Yes	97	65	0	0	97	100
No	52	35	52	100	0	0
Neoadjuvant regimen						
NACRT	83	86				
Non-NACRT	14	14				
Adjuvant therapy						
Yes	125	84	39	75	86	89
No	24	16	13	25	11	11

Podoplanin-positive CAFs were dichotomized as low or high expression according to the median value.

2.4. Statistical analysis

Associations between variables were analyzed using Fisher's exact test (for categorical variables) and the Mann–Whitney *U* test (for continuous variables). Overall survival (OS) was defined as the time from the resection date to death regardless of its cause. Disease-free survival (DFS) was defined as the time from resection to disease recurrence. Disease-specific survival (DSS) was defined as the time from resection to the date of death related to pancreatic carcinoma or last follow-up. OS, DFS, and DSS were estimated using the Kaplan–Meier method, and non-parametric group comparisons were performed using the log-rank test. Multivariate analyses were performed using the Cox proportional hazards regression model. Multivariate models were built to include factors that were found to be significant by univariate analysis. All statistical tests were two-sided, with the significance level set at 5 %. Statistical analyses were conducted using IBM SPSS Statistics for Windows (version 23.0; IBM, Armonk, NY, USA).

3. Results

3.1. Patient characteristics

The clinicopathological characteristics of all patients ($n = 149$) are summarized in Table 1. Among the 149 patients, 52 had not received neoadjuvant therapy and 97 had (chemotherapy [$n = 10$] or chemoradiation therapy [$n = 87$]). In total, 125 patients received adjuvant therapy, 86 of whom also received neoadjuvant therapy.

During the study period, 61 patients had recurrences and 39 died from pancreatic carcinoma-related causes. Six died from causes unrelated to pancreatic carcinoma. The median duration of follow-up for the patients who were alive at the time of last follow-up was 23 months (range: 4–122 months).

3.2. Tumor budding and CAFs according to neoadjuvant therapy status

The median number of tumor buds (/0.785 mm^2 field) counted with reference to the ITBCC recommendations was 26 (range: 2–372). There were significantly fewer tumor buds (/0.785 mm^2 field) in the tumors of patients who had received neoadjuvant therapy (median: 21; range: 2–112) than in those of patients who had not (median: 35; range: 7–372) ($p < 0.001$) (Fig. 3A).

The median number of tumor buds (/0.237 mm^2 field) at 40 × magnification was 14 (range: 1–259). There were significantly fewer tumor buds (/0.237 mm^2 field) in the tumors of patients who had received neoadjuvant therapy (median: 10; range 1–57) than in those of patients who had not (median: 17; range: 3–259) ($p < 0.001$) (Fig. 3B).

The median percentage of podoplanin-positive CAFs was 55 % (range: 0%–90 %). Podoplanin-positive CAFs were significantly more abundant in the tumors of patients who had received neoadjuvant therapy (median: 60 %; range: 0%–90 %) than in those of patients who had not (median: 30; range: 0–75) ($p < 0.001$) (Fig. 3C).

3.3. Tumor budding and patient outcomes in the neoadjuvant group

In patients who had received neoadjuvant therapy, the median number of tumor buds (/0.785 mm^2 field) counted with reference to the ITBCC recommendations was 26 (range: 2–372). At 20 × magnification (/0.785 mm^2 field), the median OS was lower for those

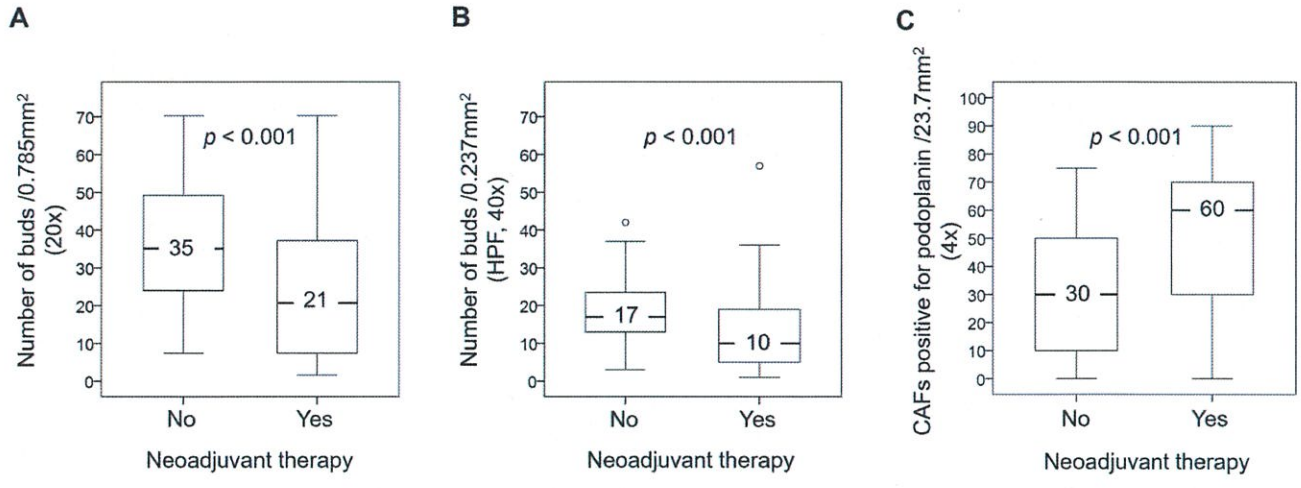

Fig. 3. Tumor budding and cancer-associated fibroblasts according to neoadjuvant therapy status. A: Number of buds/0.785 mm^2 (20 ×). B: Number of buds/0.237 mm^2 (HPF, 40 ×). C: CAFs positive for podoplanin.

with tumors in the Bd 3 category than for those with tumors in the Bd 1–2 categories (median DSS: 36 % and 58 %, respectively; $p = 0.007$) (Fig. 4A). Similar results were obtained for DSS (median DSS: 33 % and 59 %, respectively; $p = 0.004$). (Fig. 4G). Additionally, patients with tumors in the Bd 3 category tended to have worse DFS than those with tumors in the Bd 1–2 categories (median DFS: 16 % and 26 %, respectively); however, this difference was not statistically significant ($p = 0.096$) (Fig. 4D).

In patients who had received neoadjuvant therapy, when the number of tumor buds was counted at 40 × magnification (/0.237 mm^2 field), the median OS was lower for those with tumors in the Bd 3 category than it was for those with tumors in the Bd 1–2 categories (median DSS: 33 % and 59 %, respectively; $p = 0.003$) (Fig. 4B). Similar results were obtained for DSS (median DSS: 29 % and 59 %, respectively; $p = 0.001$). (Fig. 4H). Additionally, patients with tumors in the Bd 3 category tended to have worse DFS than those with tumors in the Bd 1–2 categories (median DFS: 15 % and 24 %, respectively); however, this difference was not statistically significant ($p = 0.053$) (Fig. 4E).

There were no statistically significant differences in OS or DSS between patients with tumors in the Bd 1 versus Bd 2 categories when determined by either method.

In patients treated with neoadjuvant therapy, podoplanin-positive CAFs tended to be associated with worse OS ($p = 0.055$; not significant) and DSS ($p = 0.072$; not significant) (Fig. 4C and I). There was no significant association between podoplanin-positive CAFs and DFS ($p = 0.35$) (Fig. 4F).

3.4. Clinicopathological features and patient outcomes in the neoadjuvant group

Higher T category ($p = 0.036$), lymph node metastasis ($p = 0.009$), positive resection margin ($p < 0.001$), and no adjuvant therapy ($p < 0.001$) were all found to be significantly associated with worse DSS. Higher T category ($p = 0.010$), lymph node metastasis ($p < 0.001$), positive resection margin ($p < 0.001$), lymphatic invasion ($p = 0.006$), vascular invasion ($p = 0.046$), perineural invasion ($p = 0.002$), and no adjuvant therapy ($p = 0.001$) were found to be significantly associated with worse DFS (Supplementary Table S1).

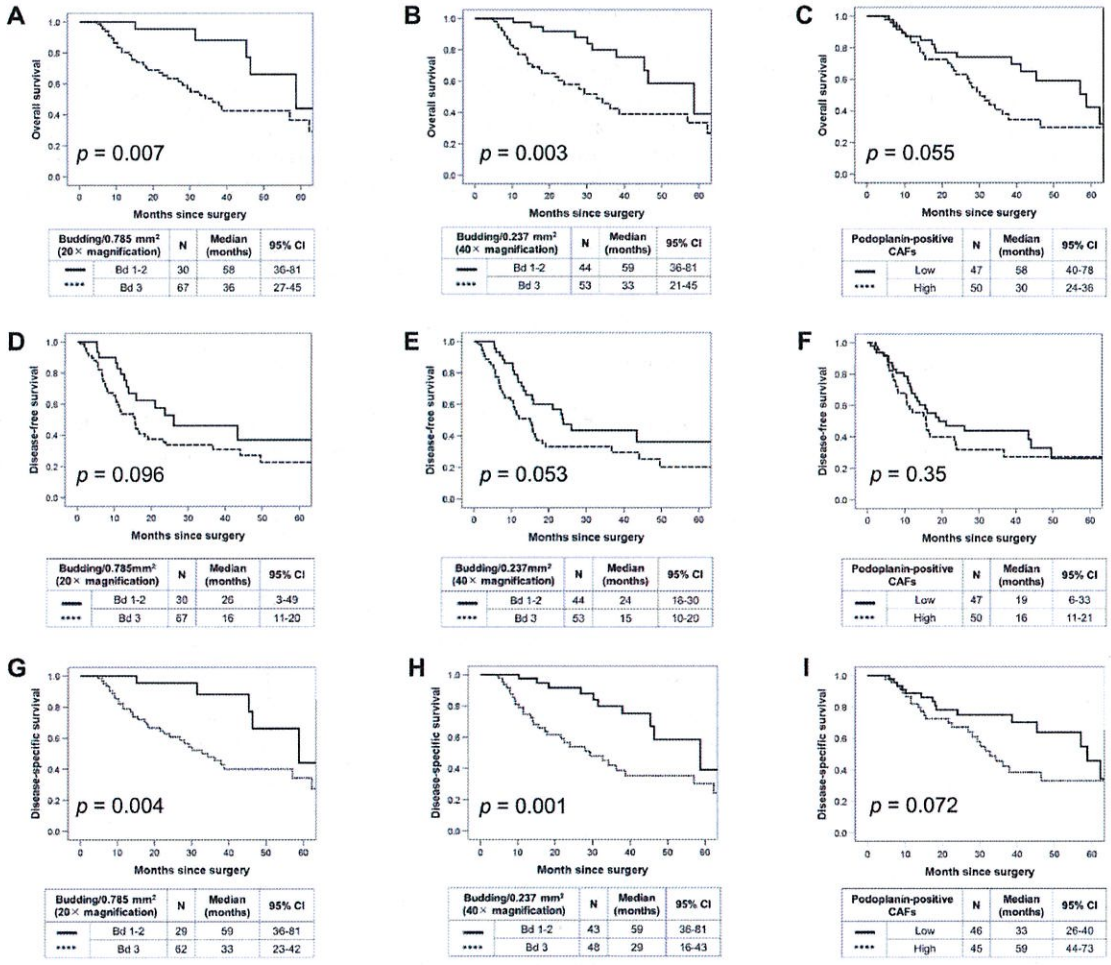

Fig. 4. Association between tumor budding, cancer-associated fibroblasts, and patient outcomes in the neoadjuvant group. Association of tumor budding with overall survival: A, B; disease-free survival: D, E; disease-specific survival: G, H. Association of podoplanin-positive CAFs with overall survival: C; disease-free survival: F; disease-specific survival: I.

3.5. Clinicopathological features, tumor budding, and podoplanin-positive CAFs in the neoadjuvant group

Table 2 summarizes the associations between clinicopathological features and tumor budding. Higher histological grade ($p < 0.001$), lymphatic invasion ($p = 0.008$), and vascular invasion ($p = 0.035$) were associated with more numerous tumor buds (/0.785 mm^2 field) when counted at 20 × and 40 × magnification. No clinicopathological features were significantly associated with podoplanin expression in CAFs (Supplementary Table S2).

3.6. Multivariate analysis of DSS

Multivariate analysis after adjusting for T category, N category, resection margin, and adjuvant therapy status revealed an association between tumor budding (/0.785 mm^2), as assessed at 20 × magnification, and shorter DSS; however, this trend was not statistically significant (hazard ratio: 2.43; $p = 0.071$) (Table 3A). Conversely, tumor budding (/0.237 mm^2 field), as assessed at 40 × magnification, was an independent prognostic factor for worse DSS (hazard ratio: 2.41; $p = 0.022$) (Table 3B).

Table 2
Associations between tumor budding and selected clinicopathologic variables in patients treated with neoadjuvant therapy.

Variables	N	Budding/0.785 mm^2 (20 ×)			Budding/0.237mm2 (HPF, 40 ×)		
		Median	range	*p*	Median	range	*p*
Age, years				0.64			0.69
<70	45	20	2–95		10	1–57	
≥70	52	21	2–112		12	1–36	
Sex				0.66			0.57
Female	45	21	2–89		11	1–33	
Male	52	20	2–112		10	1–57	
Tumor location				0.53			0.48
Head/neck	61	20	2–89		10	1–34	
Body/tail	36	26	2–112		12	1–57	
T category				0.62			0.49
T1	32	16	2–88		8	1–34	
T2	52	21	2–112		12	1–57	
T3	13	26	2–89		10	1–33	
N category				0.66			0.51
N0	55	17	2–112		9	1–57	
N1	35	22	4–89		12	2–33	
N2	7	31	2–39		13	1–23	
Resection				0.216			0.20
R0	93	21	2–112		10	1–57	
R1	4	35	16–50		19	8–27	
Histologic grade				**< 0.001**			**< 0.001**
G1	45	11	2–83		7	1–30	
G2	37	26	2–89		13	1–33	
G3	15	42	14–112		19	8–57	
Lymphatic				**0.008**			**0.013**
Negative	44	14	2–69		8	1–30	
Positive	53	26	2–112		13	1–57	
Vascular				**0.035**			**0.022**
Negative	13	7	3–83		6	2–30	
Positive	84	22	2–112		11	1–57	
Perineural				0.17			0.15
Negative	15	7	2–70		7	1–29	
Positive	82	21	2–112		11	1–57	
Evans'				0.44			0.45
Grade I	9	34	2–70		14	1–29	
Grade IIA	58	22	2–112		11	1–57	
Grade IIB	27	15	2–88		8	1–34	
Grade III	3	7	3–42		7	2–13	
CAP				0.30			0.28
Score 1	3	7	3–42		7	2–13	
Score 2	27	15	2–88		8	1–34	
Score 3	67	23	2–112		11	1–57	
MD Anderson				0.48			0.36
Score 1	3	7	3–42		7	2–13	
Score 2	94	21	2–112		10	1–57	

Abbreviations: HPF, High power field.

Table 3
Multivariate analysis of disease-specific survival.

A. Model for budding/0.785 mm^2 field (20 ×)				
Variables		HR	95 % CI	*p*
T category	T2–3 vs. T1	2.23	0.97–5.12	0.058
N category	N1–2 vs. N0	1.29	0.64–2.58	0.48
Resection margin	R1 vs. R0	2.95	0.71–12.19	0.14
Adjuvant therapy	Yes vs. No	8.91	3.00–26.35	**< 0.001**
Budding/0.785 mm^2 (20 ×)	High vs. low	2.43	0.93–6.35	0.071
B. Model for budding/0.237 mm^2 field (HPF, 40 ×)				
Variables		**HR**	**95 % CI**	***p***
T category	T2–3 vs. T1	2.27	0.99–5.12	0.053
N category	N1–2 vs. N0	1.18	0.59–2.38	0.64
Resection margin	R1 vs. R0	4.79	1.12–20.47	**0.035**
Adjuvant therapy	Yes vs. No	8.55	2.96–24.67	**< 0.001**
Budding/0.237 mm^2 (HPF, 40 ×)	High vs. low	2.41	1.13–5.13	**0.022**

Abbreviations: CI, Confidence interval; HPF, High power field; HR, Hazard ratio.

4. Discussion

Neoadjuvant therapy is increasingly being used to control local tumor spread and metastasis of PDAC. Thus, identifying prognostic factors in patients with PDAC who have received neoadjuvant therapy has assumed greater importance. Several prognostic factors for patients with PDAC who have received neoadjuvant therapy have been reported. Abnormal CA19-9 concentration and positive S factor are recognized as independent risk factors for early recurrence in patients with PDAC who have received neoadjuvant therapy [4]. TWIST1, a major epithelial–mesenchymal transition (EMT)-inducing transcription factor, is recognized as a prognostic factor for neoadjuvant chemotherapy in patients with resectable PDAC [6]. Histological evaluations of therapeutic efficacy are also important for predicting prognosis. However, the Evans system that is commonly used in Japan is problematic, with difficulty in evaluation and low interobserver concordance (kappa value) [33]. Therefore, we investigated whether tumor budding and podoplanin expression in CAFs, both of which can easily be assessed by routine pathological evaluations, are useful for predicting prognosis.

In the ITBCC recommendations, tumor budding is evaluated in hematoxylin and eosin (H&E)-stained sections. However, after neoadjuvant therapy, carcinoma cells are sparsely distributed because of degeneration, the cell boundaries are poorly defined, and the cells appear to have been assimilated into the interstitium. Therefore, we scored tumor samples using slides stained with CK AE1/AE3. Some studies have found immunohistochemistry to be superior to H&E with regards to reproducibility and interobserver agreement [34–37]. In this study, staining with CK AE1/AE3 made it easier to identify carcinoma cells and shortened the scoring time in most cases.

We evaluated tumor budding by two methods: the ITBCC "hotspot" method and the "three high power fields (3HPFs)" method. In our multivariate analysis, tumor budding as determined by the 3HPFs method was identified as an independent prognostic factor, whereas it was not a significant prognostic factor when assessed with the hotspot method. Unlike our study, the study of Petrova et al. used two different methods to assess tumor budding in hematoxylin and eosin (H&E)-stained sections, and found that both were independent prognostic factors [17]. It is assumed that this result is related to neoadjuvant therapy. In the hotspot method, inter-observer agreement can be problematic because different pathologists are likely to select fields with varying degrees of tumor budding, especially when cancer cells have undergone degeneration due to neoadjuvant therapy. Therefore, we propose that the 3HPFs method for tumor budding is more reliable and reproducible in pancreatic adenocarcinomas treated with neoadjuvant therapy. In addition, Karamitopoulou et al. reported that high interobserver agreement was observed with the 10HPFs method using CK AE1/AE3 [37]. However, unlike the 3HPFs method, the 10HPFs method is time-consuming in routine clinical practice, and may not be applicable to cases with few tumor cells remaining due to neoadjuvant therapy. In a study of patients with PDAC who did not receive neoadjuvant therapy, a two-tier prognostic stratification system with a cutoff of 10 buds/0.785 mm^2 field was reported to be more effective than a three-tier system [17]; our findings were similar. The relationship between neoadjuvant therapy and tumor budding has been investigated in perihilar cholangiocarcinoma [38] and rectal carcinoma [39], whereby abundant tumor budding was a strong indicator of poor prognosis in both, similar to our study.

In our study, podoplanin-positive CAFs were significantly more numerous in the tumors of patients who had received neoadjuvant therapy than in those of patients who had not. However, although podoplanin-positive CAFs tended to be associated with worse OS and DSS in patients receiving neoadjuvant therapy, the difference was not statistically significant, which is somewhat different to previous findings [21,40]. It has been reported that CAFs expressing podoplanin promote tumor engraftment by activating RhoA [41]. Podoplanin expression reportedly changes over time under several conditions, including co-culture with carcinoma cells, different media, and the addition of growth factors to CAF cultures [40]. It has also been reported that neoadjuvant therapy altered the collagen architecture of pancreatic carcinoma [42]. In addition, CAFs exposed to certain chemotherapeutics release chemoresistant-inducing exosomes and promote cancer proliferation and drug resistance [43]. Therefore, we considered that podoplanin expression in CAFs increases in response to changes in the tumor microenvironment caused by neoadjuvant therapy. Furthermore, a study of early-stage oral tongue cancer showed that the presence of dense CAFs is uncommon in early-stage disease [30]. The researchers suggested that

the increase in CAF density in oral tongue SCC is a time-dependent phenomenon because CAFs are thought to be recruited into the tumor from a variety of sources, not only from local resting fibroblasts [44]. In our study, the time to surgery was longer than in cases that did not receive neoadjuvant therapy; hence, the time-dependent increase in CAF density may be associated with increased podoplanin expression.

Our study had the following potential limitation. First, only one representative section was selected from each resected pancreatic carcinoma to assess tumor budding and podoplanin-positive CAFs, and thus not all slides were examined. Our findings may have been more accurate if we had stained all slides with CK AE1/AE3 and podoplanin. However, this is not feasible for routine diagnostics because it has low cost-effectiveness and is time-consuming. Second, although tumor budding has been shown to be associated with EMT in carcinoma [45], we did not investigate the association of tumor budding and EMT markers such as E-cadherin, β-catenin, and vimentin in this study.

In conclusion, tumor budding is an independent prognostic factor in PDAC patients who have received neoadjuvant therapy. Including information about tumor budding scored by the 3HPFs method in histopathological reports would improve the prognostic stratification of PDAC patients who have received neoadjuvant therapy.

Data availability

Data associated with our study has not been deposited into a publicly available repository. All data generated or analyzed during this study are included in this published article.

CRediT authorship contribution statement

Emi Ibuki: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Kyuichi Kadota:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Nachino Kimura:** Data curation. **Ryou Ishikawa:** Data curation. **Minoru Oshima:** Data curation. **Keiichi Okano:** Data curation. **Reiji Haba:** Supervision, Project administration, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank Dr. Trish Reynolds, MBBS, FRACP, and H. Nikki March, PhD, from Edanz (https://jp.edanz.com/ac) for editing a draft of this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e23928.

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