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Prognostic significance of grade of malignancy based on histopathological differentiation and Ki-67 in pancreatic ductal adenocarcinoma

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ABSTRACT

Objective: Tumor cell malignancy is indicated by histopathological differentiation and cell proliferation. Ki-67, an indicator of cellular proliferation, has been used for tumor grading and classification in breast cancer and neuroendocrine tumors. However, its prognostic significance in pancreatic ductal adenocarcinoma (PDAC) remains uncertain.

Methods: Patients who underwent radical pancreatectomy for PDAC were retrospectively enrolled, and relevant prognostic factors were examined. Grade of malignancy (GOM), a novel index based on histopathological differentiation and Ki-67, is proposed, and its clinical significance was evaluated.

Results: The optimal threshold for Ki-67 was determined to be 30%. Patients with a Ki-67 expression level > 30% rather than ≤ 30% had significantly shorter 5-year overall survival (OS) and recurrence-free survival (RFS). In multivariate analysis, both histopathological differentiation and Ki-67 were identified as independent prognostic factors for OS and RFS. The GOM was used to independently stratify OS and RFS into 3 tiers, regardless of TNM stage and other established prognostic factors. The tumor-node-metastasis-GOM stage was used to stratify survival into 5 distinct tiers, and surpassed the predictive performance of TNM stage for OS and RFS.

Conclusions: Ki-67 is a valuable prognostic indicator for PDAC. Inclusion of the GOM in the TNM staging system may potentially enhance prognostic accuracy for PDAC.

KEYWORDS Pancreatic ductal adenocarcinoma; prognosis; Ki-67; differentiation; TNM stage

Introduction

Radical surgery remains the sole viable cure for pancreatic ductal adenocarcinoma (PDAC). However, even after radical resection, patients with PDAC still face a dismal prognosis, with a 5-year overall survival (OS) rate of approximately 20%[1-5]. The poor postoperative prognosis of patients with PDAC is attributed to the advanced stage of most tumors at diagnosis and is closely associated with these tumors' aggressive biological behavior[6-8]. Precise histological grading of PDAC according to its biological behavior has immense value in predicting prognosis, guiding treatment decisions, and monitoring recurrence.

The malignancy of tumor cells is typically assessed according to the presence of cellular atypia and proliferation. Histopathological differentiation is a commonly used indicator of cellular atypia in clinical settings. The greater the degree of cellular atypia, the lower the level of differentiation. In terms of proliferation, Ki-67 is widely considered a reliable

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indicator of cellular proliferation. Expression levels of Ki-67 are correlated with recurrence risk and prognosis in various malignancies, such as gastric, prostate, and breast cancers[9-14]. Ki-67 has also been established as a crucial determinant for tumor grading and classification in pancreatic neuroendocrine neoplasms[14,15]. However, investigations of Ki-67 in PDAC have been limited in previous studies.

Herein, we conducted a retrospective analysis of data from 520 patients with PDAC who underwent curative resection. The objective of this study was to assess the potential associations of Ki-67 expression with long-term outcomes in patients with PDAC and to investigate the clinical significance of the grade of malignancy (GOM), a novel index based on histopathological differentiation and Ki-67.

Materials and methods

Study design and patients

This study was approved by the Ethics Committee of Tianjin Medical University Cancer Institute & Hospital (approval number: bc2023010). All procedures involving human participants were in accordance with the ethical guidelines outlined in the Declaration of Helsinki. Written informed consent was obtained from all patients before surgery, including a statement regarding the collection of clinicopathological data and samples for scientific purposes. A total of 836 patients in our institute's pancreatic cancer database, who underwent surgical resection for pancreatic cancer at Tianjin Medical University Cancer Institute & Hospital between January 2011 and December 2018, were eligible for this study. The inclusion criteria comprised (i) patients diagnosed with PDAC, (ii) patients who underwent curative intent pancreatic resection, (iii) patients with complete clinical and pathological examination results including Ki-67 expression, and (iv) patients who achieved satisfactory postoperative recovery and were discharged. The exclusion criteria included (i) patients with rare histopathologic subtypes, such as adenosquamous carcinomas, acinar cell carcinomas, and intraductal papillary mucinous neoplasms or mucinous cystic neoplasms with invasive cancer; (ii) patients who underwent bypass surgery or explorative laparotomy without resection; (iii) patients with macroscopic or microscopic residual tumors; (iv) patients with distant metastasis; (v) patients who died of postoperative complications; (vi) patients with a history of other malignancy; and (vii) patients lost to follow-up.

Immunohistochemical staining for Ki-67

The resected tissue specimens were fixed in 10% formaldehyde, embedded in paraffin, sectioned into slices 3–4 μm thick, and subjected to 3 rounds of xylene dewaxing for 10 min each. The specimen slides were subsequently rehydrated in a descending graded ethanol series ranging from anhydrous ethanol to distilled water. To elicit antigenic epitopes, the samples were subjected to heat treatment in a citric acid buffer (98–100°C, pH 6) in a microwave oven for 20 min before staining. Subsequently, the sections were rinsed twice with distilled water for 2 min each, then washed twice with Tris-buffered saline (TBS) for another 2 min each. After washing, the slides were incubated with a primary antibody to Ki-67 at room temperature for 60 min. Monoclonal mouse anti-human Ki-67 antigen (MIB-1; Dako, Glostrup, Denmark) was used at a dilution of 1:80 as the primary antibody. After incubation, the primary antibody was washed 3 times with TBS for 5 min each to remove any unbound antibodies. The slides were subsequently incubated with the secondary antibody anti-mouse/rabbit Ki-67 for 30 min at room temperature and visualized with an EnVision™ FLEX/HRP (Dako) system. Subsequently, the slices were subjected to 3 TBS washes for 5 min each. Subsequently, the sections were stained with diaminobenzidine tetrahydrochloride solution at room temperature for 10 min. The slides were then treated with an ascending graded ethanol series and immersed in xylene before being mounted.

Data collection

Clinicopathological data, including gender, age at surgery, preoperative serum levels of carbohydrate antigen (CA) 19-9 and carcinoembryonic antigen (CEA), tumor location, type of pancreatic resection, histopathological differentiation, Ki-67, T stage, N stage, TNM stage, lymphovascular involvement, perineural invasion, postoperative complications, and adjuvant chemotherapy were obtained from the pancreatic cancer database of our institute. During the postoperative period of hospitalization, some patients experienced complications directly associated with the surgical procedure, including hemorrhage, anastomotic leakage, pancreatic

fistulas, chyle leaks, and infections in the abdomen or at the site of incision.

The tumors were staged in accordance with the eighth edition of the Union for International Cancer Control TNM classification system. Preoperative serum tumor markers (CA19-9 and CEA) were measured within 1 week before surgery. For patients with obstructive jaundice, serum CA19-9 was re-measured after biliary drainage.

Outcome measures and statistical analysis

Survival analysis was performed with the Kaplan–Meier method and log rank test, with the following Ki-67 cutoff values: 10%, 20%, 30%, 40%, 50%, and 60%, and quartiles. The Ki-67 cutoff value with the highest χ^2 value was considered the optimal threshold of classification. All patients were categorized into 2 groups according to the optimal threshold for Ki-67. Clinicopathological feature comparison was conducted between groups, and the prognostic value of Ki-67 was assessed. GOM, a novel index based on histopathological differentiation and Ki-67 is proposed herein, and its clinical significance was evaluated. A tumor-node-metastasis-grade of malignancy (TNMG) staging system was developed by incorporating the GOM into the eighth edition of the TNM staging system, thereby enhancing the prognostic value. The discriminatory power of these 2 staging systems was evaluated with receiver operating characteristic curves, and their areas under the curve (AUCs) were compared with a Z test.

Categorical variables are presented as absolute values and relative frequencies (percentages), and were compared with the chi-square test or Fisher's exact test. Grade data were compared with the Mann-Whitney U test. OS was calculated from the date of surgery until death or the last follow-up, whereas recurrence-free survival (RFS) was defined as the time interval between surgery and tumor recurrence or the last follow-up. The date of the last follow-up was March 30, 2022. OS and RFS curves were generated with the Kaplan-Meier method. The log-rank test was used to evaluate significant differences between curves. Univariate and multivariate survival analyses were performed with Cox proportional hazards regression analysis. Significant factors in the univariate analysis were further incorporated into the multivariate analysis to determine independent prognostic factors. A two-tailed P value < 0.050 was considered statistically significant. The statistical analysis was performed with the statistical analysis program package SPSS 22.0 and MedCalc v.20.

Results

Clinicopathological features

The flowchart depicting the exclusion criteria for this study is presented in **Supplementary Figure S1**. After exclusion of 316 patients, 520 patients were considered eligible for inclusion in the study. Among the 520 patients who underwent pancreatectomy with curative intent for PDAC, 225 (42.5%) were women, and the median age was 61 years (IQR: 55–67). Most tumors were located in the pancreatic head. Pancreaticoduodenectomy was performed in 351 patients (67.5%), whereas distal pancreatectomy was performed in 169 patients (32.5%). Twenty-one patients underwent resection and reconstruction of the superior mesenteric vein/portal vein. Postoperative complications were experienced by 134 patients (25.8%), all of whom recovered after conservative treatment. Among the 265 patients (49.2%) who received postoperative adjuvant chemotherapy, 5-fluorouracil, leucovorin, gemcitabine and oxaliplatin (mFOLFIRINOX), gemcitabine and capecitabine (GX), gemcitabine and S-1 (GS), S-1, or gemcitabine was administered.

The patients were categorized into 2 groups according to Ki-67 expression: a low-expression group with a Ki-67 index of 30% or less and a high-expression group with a Ki-67 index exceeding 30%. The clinicopathological characteristics of both groups are presented in **Table 1**. Overall, the distribution of covariates between groups was nearly equivalent, with the exception of preoperative serum CEA levels and lymphovascular invasion. No significant differences were observed between groups in terms of gender, age, preoperative serum CA19-9 levels, tumor location, type of pancreatic resection, histopathological differentiation, T stage, N stage, TNM stage, perineural invasion, postoperative complications, and adjuvant chemotherapy administration. Patients in the high-expression group demonstrated a higher proportion of preoperative serum CEA levels > 5 ng/mL (35.2% *vs.* 26.8%, $P = 0.038$) and lymphovascular invasion (30.9% *vs.* 23.2%, $P = 0.048$) than those in the low-expression group.

Survival analysis of patients with PDAC

On the basis of both univariate and multivariate survival analyses, we identified the following factors as independent prognostic indicators for OS: age at surgery, preoperative serum

Table 1 Characteristics of the 520 patients with pancreatic ductal adenocarcinoma

Characteristic	Cases, *n* (%)	Ki-67 expression level		χ^2/Z	*P*
		Low-expression group, *n* (%)	High-expression group, *n* (%)		
Gender				2.082	0.149
Male	295 (56.7)	153 (53.9)	142 (60.2)		
Female	225 (43.3)	131 (46.1)	94 (39.8)		
Age (years)				0.141	0.707
< 70	444 (85.4)	244 (85.9)	200 (84.7)		
≥ 70	76 (14.6)	40 (14.1)	36 (15.3)		
Preoperative serum CA19-9 (U/mL)				−1.744	0.081
< 200	284 (54.6)	165 (50.4)	119 (50.4)		
200–1000	157 (30.2)	80 (32.6)	77 (32.6)		
> 1000	79 (15.2)	39 (13.7)	40 (16.9)		
Preoperative serum CEA (ng/mL)				4.293	0.038
≤ 5	361 (69.4)	208 (73.2)	153 (64.8)		
> 5	159 (30.6)	76 (26.8)	83 (35.2)		
Tumor location				3.418	0.064
Head	350 (67.3)	201 (70.8)	149 (63.1)		
Body and tail	170 (32.7)	83 (29.2)	87 (36.9)		
Type of pancreatectomy				3.059	0.080
PD	351 (67.5)	201 (70.8)	150 (63.6)		
DP	169 (32.5)	83 (29.2)	86 (36.4)		
Histopathologic differentiation				1.014	0.314
Well and moderate	208 (40.0)	108 (38.0)	100 (42.4)		
Poor	312 (60.0)	176 (62.0)	136 (57.6)		
T stage				3.285	0.350
T1	79 (15.2)	48 (16.9)	31 (13.1)		
T2	278 (53.5)	155 (54.6)	123 (52.1)		
T3	149 (28.7)	73 (25.7)	76 (32.2)		
T4	14 (2.7)	8 (2.8)	6 (2.5)		
N stage				2.979	0.225
N0	244 (46.9)	143 (50.4)	101 (42.8)		
N1	124 (23.8)	64 (22.5)	60 (25.4)		
N2	152 (29.2)	77 (27.1)	75 (31.8)		
TNM stage				4.489	0.106
I	168 (32.3)	103 (36.3)	65 (27.5)		
II	191 (36.7)	98 (34.5)	93 (39.4)		
III	161 (31.0)	83 (29.2)	78 (33.1)		

Table 1 Continued

Characteristic	Cases, *n* (%)	Ki-67 expression level		χ^2/Z	*P*
		Low-expression group, *n* (%)	High-expression group, *n* (%)		
Perineural invasion				0.559	0.455
Absent	183 (35.2)	104 (36.6)	79 (33.5)		
Present	337 (64.8)	180 (63.4)	157 (66.5)		
Lymphovascular invasion				3.895	0.048
Absent	381 (73.3)	218 (76.8)	163 (69.1)		
Present	139 (26.7)	66 (23.2)	73 (30.9)		
Postoperative complications				0.590	0.442
Present	134 (25.8)	77 (27.1)	57 (24.2)		
Absent	386 (74.2)	207 (72.9)	179 (75.8)		
Postoperative adjuvant chemotherapy regimens				4.439	0.350
None	255 (49.0)	131 (46.1)	124 (52.5)		
mFOLFIRINOX	66 (12.7)	41 (14.4)	25 (10.6)		
GS/GX	109 (21.0)	58 (20.4)	51 (21.6)		
S-1	45 (8.7)	25 (8.8)	20 (8.5)		
Gemcitabine	45 (8.7)	29 (10.2)	16 (6.8)		

CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; DP, distal pancreatectomy; PD, pancreaticoduodenectomy.

CA19-9 level, TNM stage, postoperative adjuvant chemotherapy, postoperative complications, histopathologic differentiation, and Ki-67 expression level (**Table 2**). Patients in the low-expression group had significantly higher OS rates than those in the high-expression group (5-year OS: 25.0% *vs.* 17.0%, $P < 0.001$) (**Figure 1A, B**).

Similarly, high expression of Ki-67 (HR: 1.454, 95% CI: 1.185–1.781, $P < 0.001$), poor histopathological differentiation (HR: 1.632, 95% CI: 1.323–12.012, $P < 0.001$), high preoperative serum CA19-9 levels, advanced TNM stage, and absence of postoperative adjuvant chemotherapy were identified as unfavorable independent prognostic factors for RFS (**Table 3**). Notably, patients in the low-expression group exhibited a significantly superior RFS rate to those in the high-expression group, with a 5-year RFS of 16.5% *vs.* 10.0%, respectively ($P < 0.001$) (**Figure 1C, D**).

Establishment of the malignancy grading system

Patients in the low-expression group had significantly higher OS and RFS rates than those in the high-expression group, regardless of histopathological differentiation (**Supplementary Figure S2**). Survival curves according to histopathological differentiation and Ki-67 expression levels are presented in **Figure 2**. The prognosis for patients with well and moderately differentiated tumors but a Ki-67 index > 30% was comparable to that of patients with poorly differentiated tumors and a Ki-67 index ≤ 30%; significantly worse than that of patients with well and moderately differentiated tumors and a Ki-67 index ≤ 30%; and better than that of patients with poorly differentiated tumors and a Ki-67 index > 30% (**Figure 2**). On the basis of these findings, we proposed a malignancy grading system that takes histopathological differentiation and Ki-67 into account. The low-grade category refers to well and moderately differentiated cancers with a Ki-67 index ≤ 30%; the middle-grade category includes well and moderately differentiated cancers with a Ki-67 index > 30% or poorly differentiated cancers with a Ki-67 index ≤ 30%; and the high-grade category comprises poorly differentiated cancers with a Ki-67 index > 30% (**Figure 3**). Representative Ki-67 immunohistochemical staining images of PDAC with different differentiation status are shown in **Figure 4**. The GOM was able to stratify patients into 3 distinct groups with significant differences

Table 2 Univariate and multivariate analyses of factors associated with OS

Characteristics	*n*	MST	5-year OS (%)	Univariate analysis		Multivariate analysis	
				HR (95% CI)	*P*	HR (95% CI)	*P*
Gender							
Male	295	20.0	19.5	1 (ref)			
Female	225	24.0	18.8	0.912 (0.736–1.130)	0.400		
Age at surgery							
< 70	444	22.0	20.3	1 (ref)		1 (ref)	
≥ 70	76	16.0	12.4	1.421 (1.067–1.892)	0.016	1.611 (1.199–2.167)	0.002
Preoperative serum CA19-9 (U/mL)							
< 200.0	284	25.0	21.8	1 (ref)		1 (ref)	
200.0–1000.0	157	20.0	18.1	1.322 (1.041–1.680)	0.022	1.323 (1.033–1.695)	0.027
> 1000.0	79	16.0	15.0	1.875 (1.398–2.514)	< 0.001	1.524 (1.114–2.084)	0.008
Preoperative serum CEA (ng/mL)							
≤ 5	361	24.0	22.0	1 (ref)		1 (ref)	
> 5	159	18.0	12.1	1.386 (1.105–1.738)	0.005	1.154 (0.898–1.483)	0.264
Tumor location							
Head	350	21.0	20.2	1 (ref)			
Body and tail	170	21.0	17.4	1.021 (0.813–1.282)	0.859		
Type of pancreatectomy							
PD	351	21.0	20.0	1 (ref)			
DP	169	21.0	17.7	1.049 (0.836–1.318)	0.678		
Histopathologic differentiation							
Well and moderate	208	32.0	27.0	1 (ref)		1 (ref)	
Poor	312	16.0	14.1	1.852 (1.481–2.318)	< 0.001	1.875 (1.488–2.363)	< 0.001
TNM stage							
I	168	35.0	30.7	1 (ref)		1 (ref)	
II	191	20.0	18.0	1.665 (1.269–2.184)	< 0.001	1.371 (1.036–1.816)	0.027
III	161	14.0	8.0	2598 (1.98–3.407)	< 0.001	2.496 (1.84–3.375)	< 0.001
Perineural invasion							
Absent	183	28.8	24.0	1 (ref)		1 (ref)	
Present	337	12.2	20.0	1.377 (1.098–1.727)	0.006	1.123 (0.890–1.417)	0.329
Lymphovascular invasion							
Absent	381	20.4	24.0	1 (ref)		1 (ref)	
Present	139	16.0	17.0	1.383 (1.097–1.743)	0.006	1.160 (0.893–1.506)	0.266
Ki-67 expression level							
Low expression	284	25.0	25.0	1 (ref)		1 (ref)	
High expression	236	11.8	17.0	1.669 (1.350–2.063)	< 0.001	1.707 (1.371–2.126)	< 0.001

Table 2 Continued

Characteristics	*n*	MST	5-year OS (%)	Univariate analysis		Multivariate analysis	
				HR (95% CI)	*P*	HR (95% CI)	*P*
Postoperative adjuvant chemotherapy							
No	265	16.0	27.1	1 (ref)		1 (ref)	
Yes	255	26.0	11.4	1.813 (1.466–2.242)	< 0.001	0.573 (0.432–0.667)	< 0.001
Postoperative complications							
Absent	386	24.0	21.4	1 (ref)		1 (ref)	
Present	134	16.0	12.2	1.539 (1.215–1.950)	< 0.001	1.434 (1.120–1.837)	0.004

CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; DP, distal pancreatectomy; PD, pancreaticoduodenectomy; ref, reference category; HR, hazard ratio; OS, overall survival.

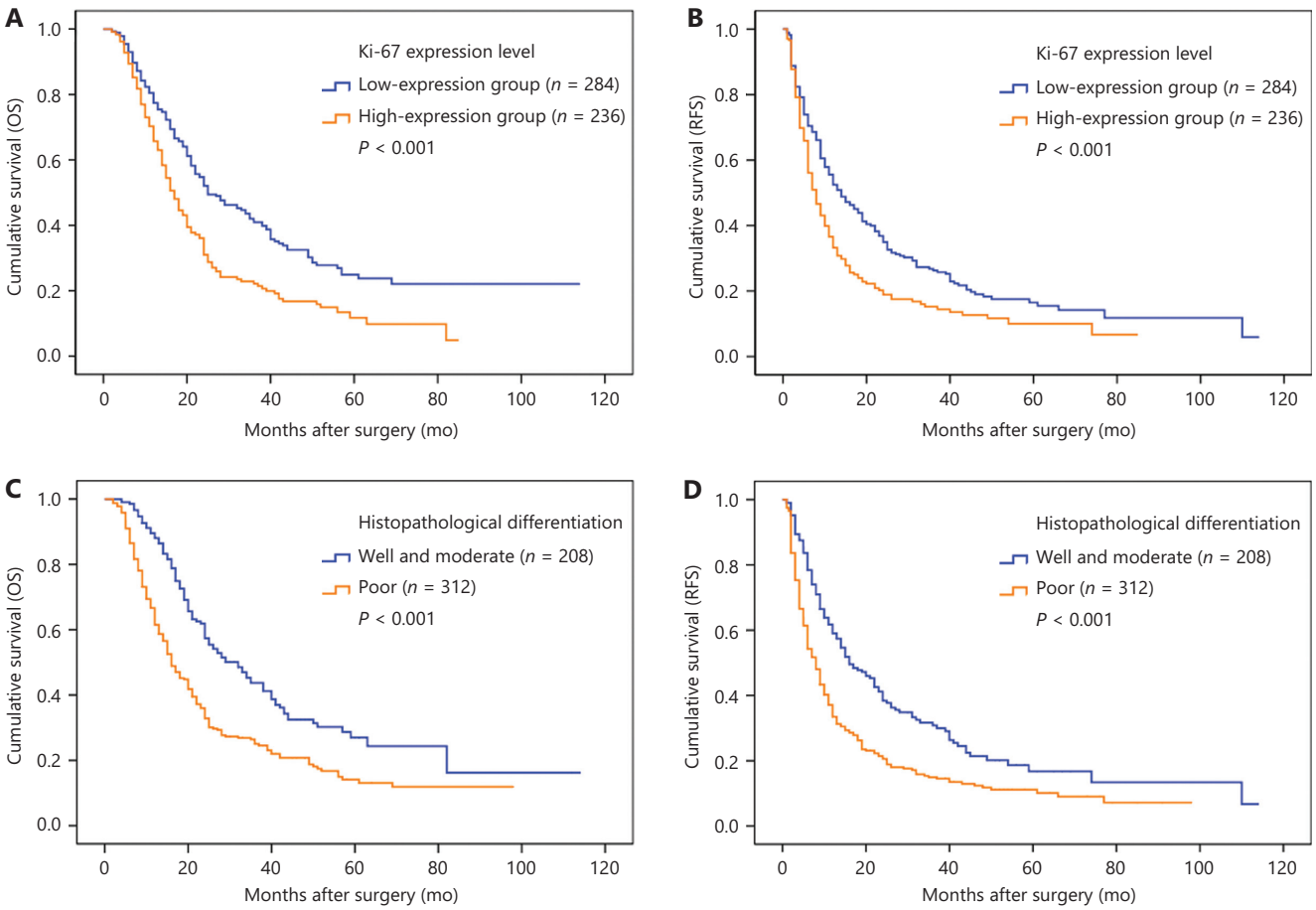

Figure 1 Kaplan–Meier curves of overall survival (OS) and recurrence-free survival (RFS) according to the Ki-67 index or histopathological differentiation. (A) OS curves according to Ki-67 expression level. (B) RFS curves according to Ki-67 expression level. (C) OS curves according to histopathological differentiation. (D) RFS curves according to histopathological differentiation.

Table 3 Univariate and multivariate analyses of factors associated with RFS

Characteristics	*n*	MRT	5-year RFS (%)	Univariate analysis		Multivariate analysis	
				HR (95% CI)	*P*	HR (95% CI)	*P*
Gender							
Male	295	10.0	12.9	1 (ref)			
Female	225	12.0	14.5	0.931 (0.763–1.136)	0.483		
Age at surgery							
< 70	444	11.0	13.6	1 (ref)			
≥ 70	76	8.0	14.5	1.142 (0.864–1.509)	0.352		
Preoperative serum CA19-9 (U/mL)							
< 200.0	284	14.0	18.6	1 (ref)		1 (ref)	
200.0–1000.0	157	10.0	8.9	1.363 (1.090–1.703)	0.007	1.310 (1.041–1.649)	0.021
≥ 1000.0	79	6.0	–	2.182 (1.658–2.871)	< 0.001	1.806 (1.346–2.423)	< 0.001
Preoperative serum CEA (ng/mL)							
≤ 5	361	12.0	15.2	1 (ref)		1 (ref)	
> 5	159	8.0	9.8	1.382 (1.118–1.708)	0.003	1.158 (0.915–1.464)	0.222
Tumor location							
Head	350	10.0	13.5	1 (ref)			
Body and tail	170	10.0	12.9	0.993 (0.804–1.227)	0.951		
Type of pancreatectomy							
PD	351	11.0	13.5	1 (ref)			
DP	169	10.0	13.1	1.027 (0.831–1.268)	0.808		
Histopathologic differentiation							
Well and moderate	208	16.0	16.8	1 (ref)		1 (ref)	
Poor	312	8.0	11.1	1.705 (1.388–2.095)	< 0.001	1.632 (1.323–2.012)	< 0.001
TNM stage							
I	168	20.0	22.2	1 (ref)		1 (ref)	
II	191	11.0	15.3	1.448 (1.128–1.858)	0.004	1.191 (0.921–1.540)	0.182
III	161	6.0	3.4	2.519 (1.960–3.237)	< 0.001	2.141 (1.623–2.826)	< 0.001
Perineural invasion							
Absent	183	13.0	21.1	1 (ref)		1 (ref)	
Present	337	9.0	8.5	1.404 (1.136–1.736)	0.002	1.154 (0.929–1.435)	0.196
Lymphovascular invasion							
Absent	381	12.0	15.7	1 (ref)		1 (ref)	
Present	139	6.0	8.4	1.596 (1.289–1.977)	< 0.001	1.061 (0.936–1.349)	0.625
Ki-67 expression level							
Low expression	284	14.0	16.5	1 (ref)		1 (ref)	
High expression	236	8.0	10.0	1.489 (1.222–1.816)	< 0.001	1.453 (1.185–1.781)	< 0.001

Table 3 Continued

Characteristics	*n*	MRT	5-year RFS (%)	Univariate analysis		Multivariate analysis	
				HR (95% CI)	*P*	HR (95% CI)	*P*
Postoperative adjuvant chemotherapy							
No	265	8.0	10.9	1 (ref)		1 (ref)	
Yes	255	13.0	16.2	1.367 (1.123–1.663)	0.002	0.734 (0.601–0.896)	0.002
Postoperative complications							
Absent	386	12.0	15.2	1 (ref)		1 (ref)	
Present	134	7.0	8.6	1.380 (1.105–1.723)	0.004	1.198 (0.952–1.507)	0.124

CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; DP, distal pancreatectomy; PD, pancreaticoduodenectomy; ref, reference category; HR, hazard ratio; RFS, recurrence-free survival.

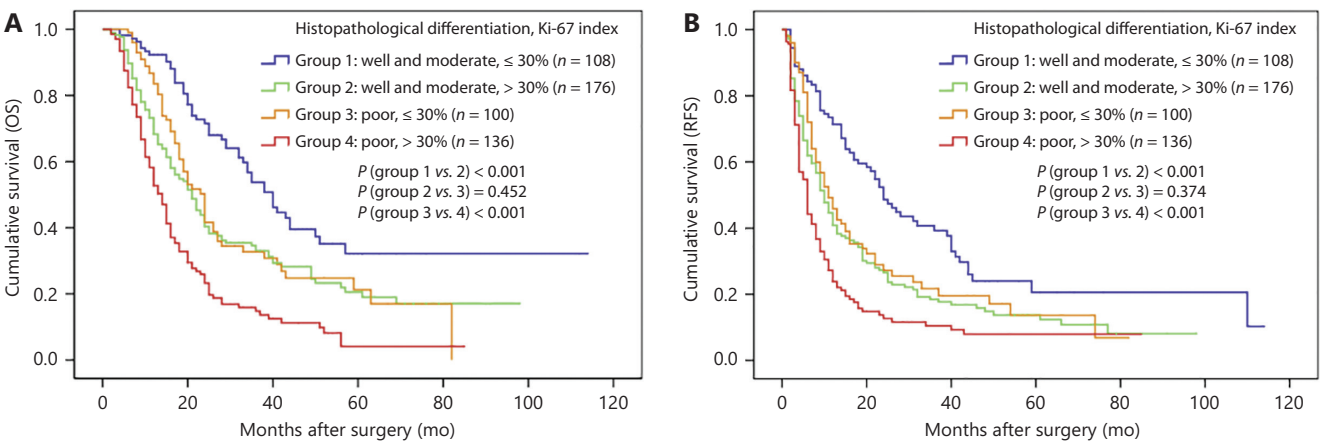

Figure 2 Survival curves based on histological differentiation and Ki-67 index. The survival of patients with well and moderately differentiated tumors with high expression of Ki-67 was comparable to that of patients with poorly differentiated tumors with low expression of Ki-67. (A) Overall survival. (B) Recurrence-free survival.

in survival (**Figure 5**). The median OS of patients with low-grade, middle-grade, and high-grade tumors were 40.0, 21.0, and 14.0 months, respectively, and the HRs (95% CIs) were 1.0 (reference), 1.827 (1.341–2.488), and 3.372 (2.421–4.698), respectively ($P < 0.001$). The median RFS among patients with low-grade, middle-grade, and high-grade tumors were 24.0, 10.0, and 6.0 months, respectively, and the HRs (95% CIs) were 1.0 (reference), 1.733 (1.316–2.282), and 2.745 (2.026–3.721), respectively ($P < 0.001$). To determine whether the GOM could be used to independently stratify patients according to survival, we conducted a multivariate analysis. The GOM was significantly associated with OS and RFS, independently

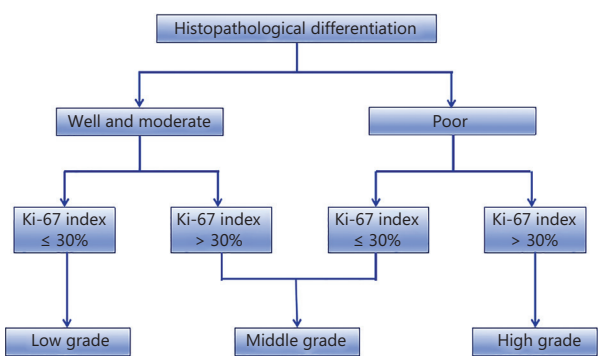

Figure 3 The proposed malignancy grading system and its algorithm.

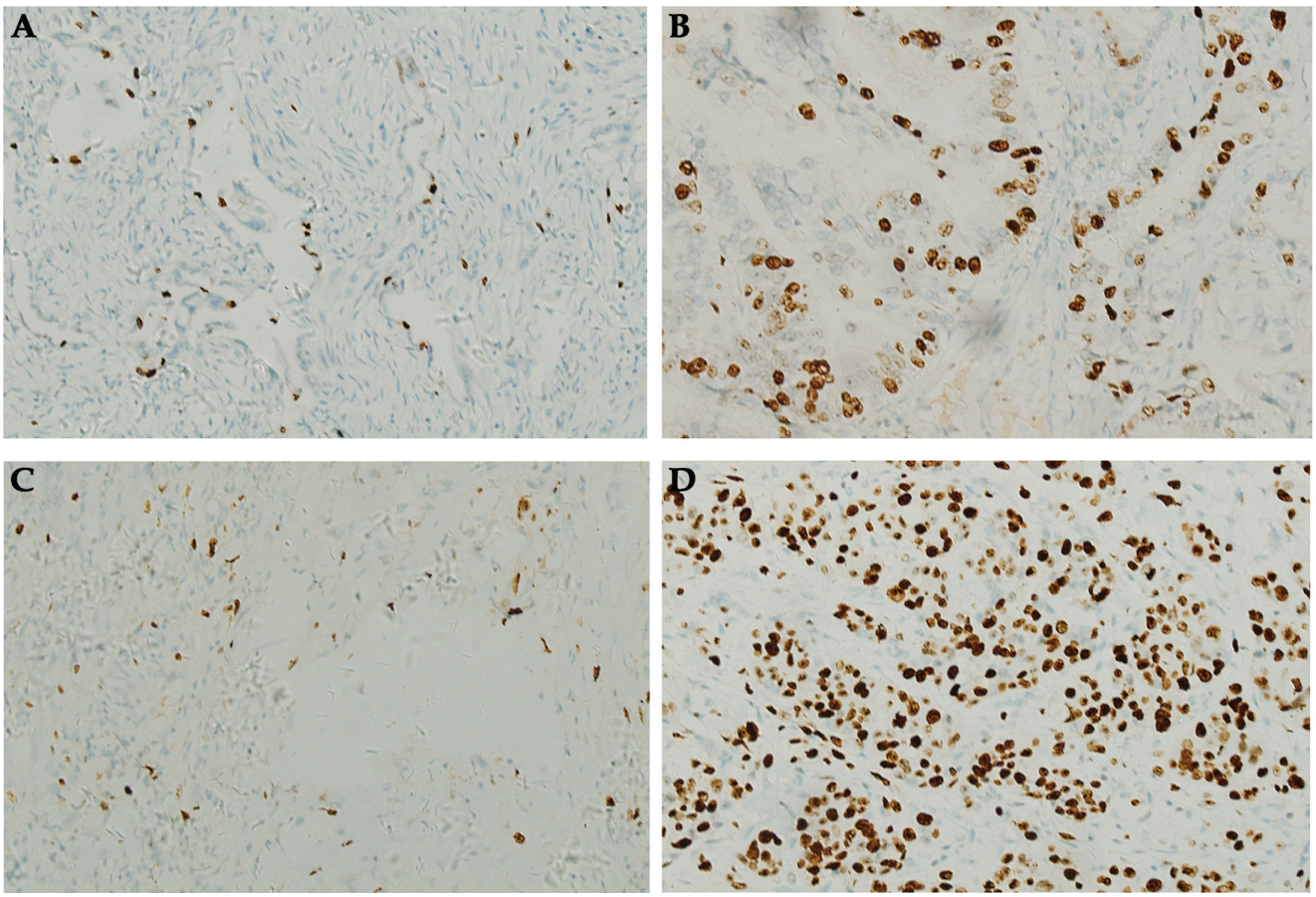

Figure 4 Ki-67 immunohistochemical staining in pancreatic ductal adenocarcinoma. (A) Well-differentiated tumor with a Ki-67 index of 20%. (B) Moderately differentiated tumor with a Ki-67 index of 60%. (C) Poorly differentiated tumor with a Ki-67 index of 5%. (D) Poorly differentiated tumor with a Ki-67 index of 70% (X+number).

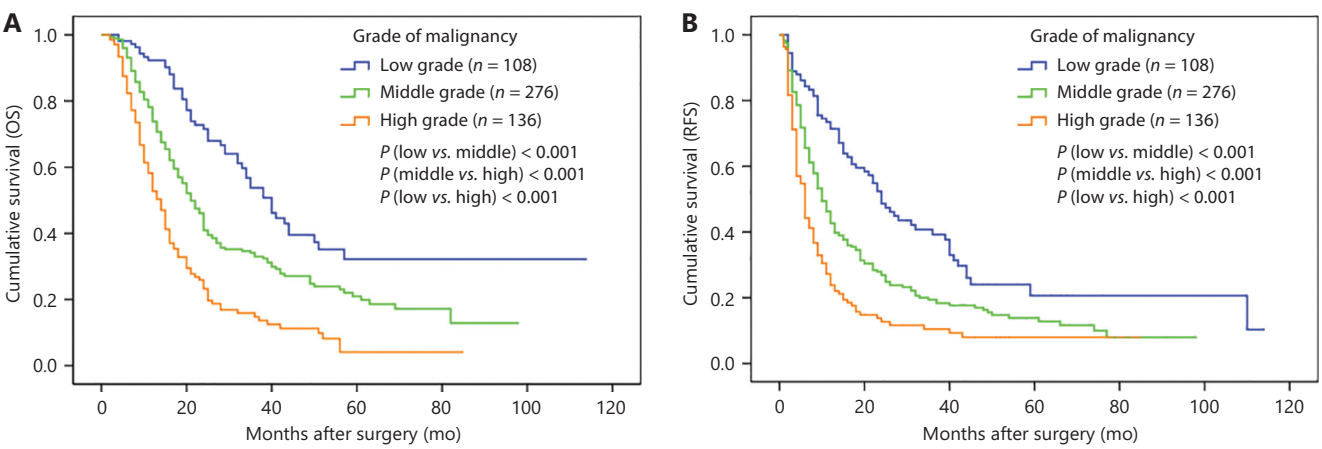

Figure 5 Survival curves according to the grade of malignancy (GOM). The GOM stratified patients into 3 distinct groups with significantly differing prognosis. (A) Overall survival. (B) Recurrence-free survival.

of TNM stage and other well-established prognostic factors (**Table 4**).

Incorporation of the GOM into the eighth edition of the TNM staging system

We subsequently used stratified analysis to investigate whether the GOM remained a significant predictor for different TNM stages. Regardless of the TNM stage, the GOM significantly predicted OS and RFS (**Supplementary Table S1**). The OS and RFS rates were comparable between patients with stage II low-grade and stage I middle-grade disease. Moreover, no statistically significant differences were observed in the OS and RFS rates between patients with stage III middle-grade and stage II high-grade disease. Similarly, we observed no significant differences in survival rates among patients with stage III low-grade, stage II middle-grade, and stage I high-grade disease (**Figure 6**). On the basis of these results, we integrated the GOM into the eighth edition of the TNM staging system, establishing a TNMG staging system. The TNMG staging system and its corresponding algorithm are presented in **Figure 7**. In accordance with the algorithm, patients were categorized into 5 stages: TNMG stage I (n = 49), stage IIa (n = 124), stage IIb (n = 157), stage IIIa (n = 137), and stage IIIb (n = 53). Survival of patients was clearly distinguished by TNMG stage (**Figure 8**). The median OS for patients with TNMG stage I, IIa, IIb, IIIa, and IIIb were 57.0, 28.0, 23.0,

Table 4 Multivariate survival analysis examining the prognostic value of the malignancy grading system in the context of known independent prognostic factors

Variable	Category	HR (95% CI)	*P*
Multivariate analysis for OS			
GOM	High *vs.* low	3.302 (2.350–4.641)	< 0.001
	Middle *vs.* low	1.967 (1.433–2.700)	< 0.001
TNM stage	III *vs.* I	2.370 (1.791–3.137)	< 0.001
	II *vs.* I	1.401 (1.065–1.843)	0.016
Preoperative serum CA19-9 (U/mL)	≥ 1000.0 *vs.* < 200	1.622 (1.207–2.180)	0.001
	200–1000 *vs.* < 200	1.364 (1.068–1.741)	0.013
Postoperative adjuvant chemotherapy	Yes *vs.* no	0.539 (0.434–0.669)	< 0.001
Age at surgery	≥ 70 *vs.* < 70	1.657 (1.235–2.223)	0.001
Postoperative complications	Present *vs.* absent	1.443 (1.126–1.849)	0.004
Multivariate analysis for RFS			
GOM	High *vs.* low	2.596 (1.904–3.538)	< 0.001
	Middle *vs.* low	1.751 (1.324–2.315)	< 0.001
TNM stage	III *vs.* I	2.244 (1.736–2.901)	< 0.001
	II *vs.* I	1.233 (0.957–1.588)	0.106
Preoperative serum CA19-9 (U/mL)	≥ 1000.0 *vs.* < 200	1.905 (1.442–2.516)	< 0.001
	200–1000 *vs.* < 200	1.350 (1.078–1.693)	0.009
Postoperative adjuvant chemotherapy	Yes *vs.* no	0.748 (0.613–0.913)	0.004
Age at surgery	≥ 70 *vs.* < 70	1.226 (0.924–1.625)	0.157
Postoperative complication	Present *vs.* absent	1.207 (0.958–1.520)	0.110

CA19-9, carbohydrate antigen 19-9; HR, hazard ratio; OS, overall survival; RFS, recurrence-free survival; GOM, grade of malignancy.

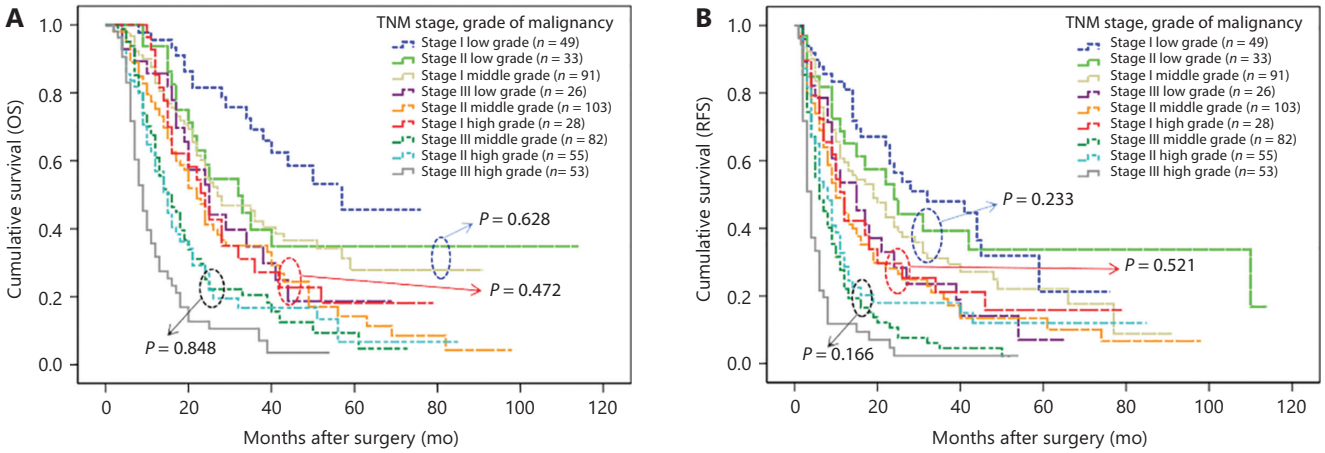

Figure 6 Survival curves based on TNM stage and the grade of malignancy (GOM). Survival was comparable between patients with stage II low-grade and stage I middle-grade disease. No statistically significant differences in survival were observed between patients with stage III middle-grade and stage II high-grade disease. Similarly, no significant disparities in survival were observed among patients with stage III low-grade, stage II middle-grade, and stage I high-grade disease. (A) Overall survival. (B) Recurrence-free survival.

15.0, and 9.0 months, respectively, and the HRs (95% CIs) were 1.0 (reference), 1.913 (1.124–3.257), 2.863 (1.714–4.783), 4.556 (2.725–7.617), and 8.699 (4.974–15.214), respectively (overall $P < 0.001$). The median RFS for patients with TNMG stage I, IIa, IIb, IIIa, and IIIb were 32.0, 22.0, 12.0, 7.0, and 4.0 months, respectively, and the HRs (95% CIs) were 1.0 (reference), 1.382 (0.893–2.139), 2.084 (1.372–3.166), 3.279 (2.153–4.995), and 6.038 (3.749–9.723), respectively (overall $P < 0.001$).

TNM stage	Grade of malignancy		
	Low	Middle	High
I	I	IIa	IIb
II	IIa	IIb	IIIa
III	IIb	IIIa	IIIb
IV	IV		

Figure 7 Proposed tumor-node-metastasis-grade of malignancy (TNMG) staging system for PDAC and its algorithm.

The predictive performance of the TNMG staging system was compared with that of the TNM staging system with time-dependent AUCs for each system. The 1-year, 3-year, and 5-year time-dependent AUCs of the TNMG staging system were significantly higher than those of the TNM staging system, thereby indicating superior prognostic discrimination power (**Figure 9**).

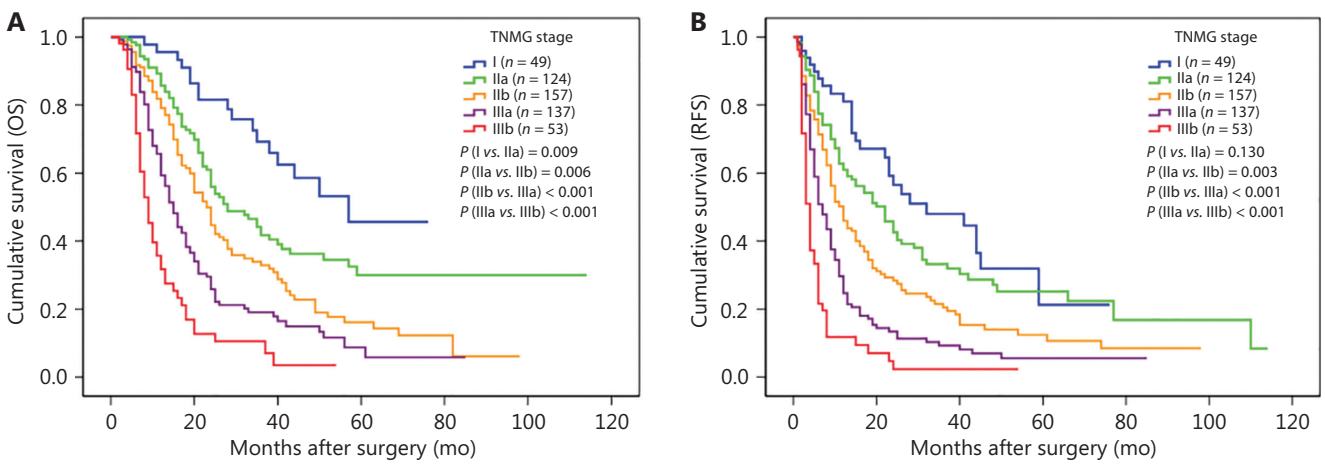

Figure 8 Survival curves according to the TNMG stage. (A) Overall survival. (B) Recurrence-free survival.

Stratified survival analysis based on the GOM

Patients with PDAC who received adjuvant chemotherapy after surgery had significantly longer OS with than those who did not receive chemotherapy (median OS: 26.0 *vs.* 16.0 months, $P < 0.001$). The analysis stratified by the GOM revealed that postoperative adjuvant chemotherapy did not confer a survival benefit in patients with low-grade tumors, but prolonged OS in those with middle- to high-grade malignancies (**Supplementary Table S2**, **Supplementary Figure S3**).

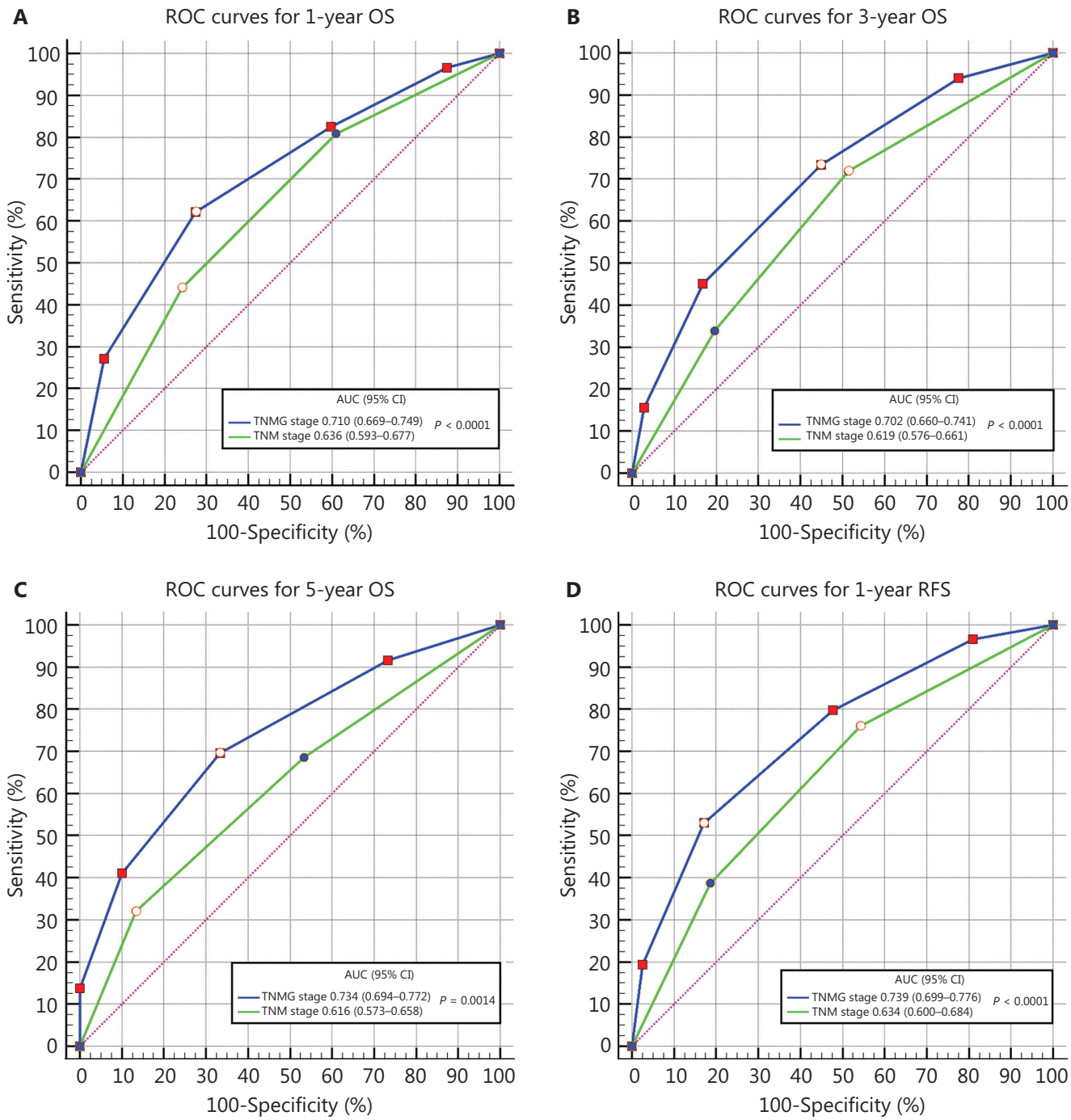

Figure 9 Continued

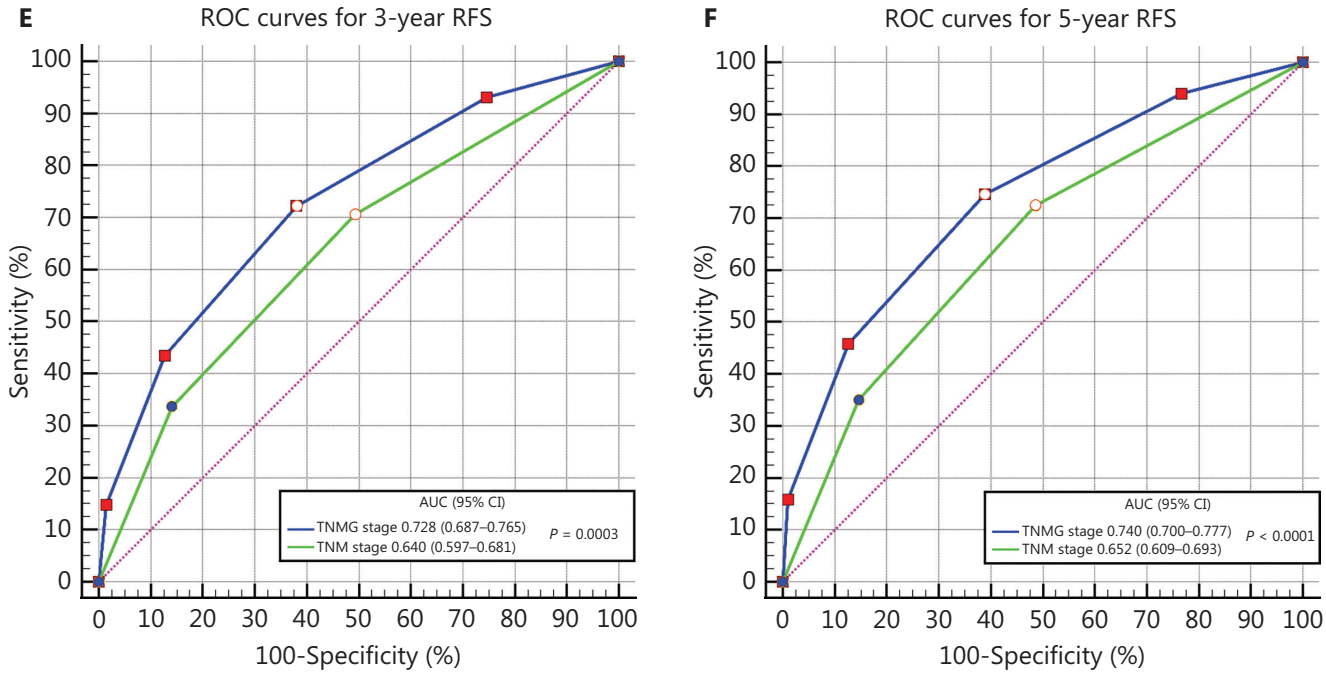

Figure 9 Receiver operating characteristic curves, demonstrating superior AUC values for the proposed TNMG stages compared with the TNM stages for OS and RFS at 1-, 3-, and 5-year after surgery. (A) OS at 1 year, (B) OS at 3 years, (C) OS at 5 years, (D) RFS at 1 year, (E) RFS at 3 years, and (F) RFS at 5 years.

Discussion

The biological behavior of malignancies is reflected partly by cell differentiation and proliferation, and Ki-67 is the most commonly used indicator for assessing cell proliferation. High expression levels of Ki-67 have been associated with an increased risk of recurrence in various tumors, such as neuroendocrine tumors[16-18], breast cancer[12,13], and gastrointestinal stromal tumors[19]. The poor prognosis of PDAC is closely associated with its aggressive biological behavior; however, few studies have focused on Ki-67[20-23]. In the present study, we investigated the expression of Ki-67 in patients with PDAC who underwent curative resection. Our results confirmed that the Ki-67 index is an independent prognostic factor for PDAC. Furthermore, GOM, a novel index based on Ki-67 and histopathological differentiation, is proposed to better assess the aggressive biological behavior of PDAC. The GOM can be used to independently stratify OS and RFS into 3 tiers, regardless of TNM stage or other established prognostic factors. Additionally, we incorporated the GOM into the TNM staging system to establish a new TNMG staging system that can stratify survival into 5 tiers, and outperforms the traditional TNM staging system in predicting OS and RFS.

Histopathological differentiation is a crucial factor in the prognosis of various malignancies, including PDAC[24-26]. Tumor differentiation serves as a standard reflecting the degree of malignancy at the histological level. In this study, we confirmed that histopathological differentiation independently correlated with OS and RFS in patients with PDAC. Patients with well-differentiated or moderately differentiated tumors had better prognosis than those with poorly differentiated tumors. However, histopathological differentiation does not necessarily reflect cell proliferation. The malignancy of tumors is closely correlated with the rate of cell proliferation. A higher rate of cell proliferation leads to faster tumor growth and increased malignancy. Ki-67 is a nucleoprotein that plays a crucial role in cell proliferation and ribosomal RNA transcription. Its expression is observed during the G1, S, G2, and M phases of the cell cycle but not during the quiescent G0 phase[27,28]. Therefore, Ki-67 expression serves as an indicator of cellular proliferation, with higher levels indicating greater proportions of actively dividing cells. Ki-67 is considered the cornerstone for grading neuroendocrine tumors and a crucial marker for luminal classification in breast cancer[29,30]. Numerous studies have demonstrated that high expression of Ki-67 is associated with unfavorable prognosis across various

malignancies[12,13,16-19]. Regarding PDAC, limited research has been conducted on Ki-67. Pergolini and colleagues[20] have demonstrated that Ki-67 is an independent predictor of poor disease-free and disease-specific survival. Several studies have indicated that a high Ki-67 index has prognostic value in PDAC[21-23]. Our findings are consistent with those of previous studies, indicating that both histopathological differentiation and Ki-67 expression level are independent prognostic factors for OS and RFS. Notably, even among patients with the same degree of histopathological differentiation, those with a Ki-67 index > 30% had significantly lower rates of OS and RFS than those with a Ki-67 index ≤ 30%. Therefore, combining histopathological differentiation and Ki-67 to classify the degree of malignancy at the histological level is essential. Our proposed GOM can be used to independently stratify patient survival regardless of TNM stage or other established prognostic factors. This GOM reflects both cellular atypia and proliferation at the histological level, and consequently is a reliable indicator of aggressive biological behavior.

The TNM staging system is the internationally recognized standard for cancer staging and prognostication[31-33]. However, its application in PDAC has limitations in predicting prognosis, because of its reliance on anatomical factors—which may reflect tumor burden but not necessarily biological behavior—instead of biological factors[34,35]. The poor prognosis of PDAC is closely associated with its aggressive biological behavior. Our findings demonstrated that even among patients with the same TNM stage, the GOM remained a significant predictor of survival, and low-grade tumors were associated with significantly better prognosis than middle- and high-grade tumors. Revising the TNM staging system by incorporating our proposed GOM may compensate for its limitations in prognostic evaluation. A previous study has proposed a refined prognostic staging system for resected pancreatic cancer through modified stage grouping and inclusion of tumor grade, and confirmed that this refined staging system outperforms the TNM staging system[8]. In this study, we classified patients into 5 subgroups according to the TNMG staging system, which was superior to the traditional TNM staging system in predicting OS and RFS. The TNMG staging system incorporates tumor burden and biological behavior factors, thus resulting in more accurate prognostication than the TNM staging system.

The GOM is determined by the degree of cellular differentiation and proliferation, which are closely associated with chemotherapy sensitivity[36,37]. Therefore, the GOM can serve as a valuable indicator for postoperative adjuvant therapy. In this study, although postoperative adjuvant chemotherapy did not confer a survival advantage in patients with low-grade malignancy, it significantly increased OS in patients with middle to high grade malignancies. For the latter group, combination chemotherapy involving 2 or more agents is recommended, whereas single-agent chemotherapy may be sufficient for those with low-grade malignancies.

This study has several limitations. First, Ki-67 and histopathological differentiation were obtained from surgical specimens, thus limiting their applicability to locally advanced or metastatic PDAC. However, the expression levels of Ki-67 in biopsy tissues and surgical specimens are comparable and significantly correlated with the prognosis of pancreatic neuroendocrine tumors[38-40]. Ascertaining the differentiation and Ki-67 index of biopsy tissues is imperative, because the GOM of such samples has substantial value in determining an effective treatment plan. Low-grade tumors may not significantly benefit from chemotherapy, and upfront surgery may be a more viable option. In contrast, middle- and high-grade tumors are often responsive to chemotherapy; consequently, neoadjuvant chemotherapy followed by surgery may be a more appropriate course of action. Second, the determination of the optimal cutoff value for the Ki-67 index lacks a standardized criterion and requires extensive investigation in large sample sizes. Third, other proliferative markers, such as PCNA and MCM-2, were not explored. The relationships between these proliferative indicators and Ki-67 were also not evaluated. Because no other proliferative indicators were included in our pathology report, which proliferative indicators best reflect the proliferation of pancreatic cancer cells must be investigated in the future. Finally, this study was a single-center retrospective analysis. Therefore, our findings require validation on the basis of data from other institutions, and further large-scale multicenter prospective studies are necessary.

Conclusions

A Ki-67 index > 30% is significantly associated with inferior OS and RFS in patients diagnosed with PDAC. The GOM, determined by histopathological differentiation and Ki-67 expression, may serve as a predictive biomarker for the prognosis of patients with PDAC who have undergone curative resection. Incorporating the GOM into the staging system enhances the accuracy of TNM staging in predicting prognosis, and additionally can be used to guide postoperative

adjuvant therapy, which should be considered in the selection of therapeutic regimens.

Conflicts of interest statement

No potential conflicts of interest are disclosed.

Author contributions

Conceived and designed the analysis: Yuexiang Liang, Guannan Sheng, Song Gao, Jihui Hao.
Collected the data: Yuexiang Liang, Shaofei Chang, Quan Man, Yu Guo, Haohan Guo, Zhifei Li, Yiping Zou.
Contributed data or analysis tools: Yiping Zou, Yu Guo, Hanhan Guo, Shaofei Chang.
Performed the analysis: Yiping Zou, Zhifei Li.
Wrote the paper: Yuexiang Liang, Guannan Sheng, Song Gao.

Data availability statement

The data that support the findings of this study are available from the corresponding author.

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