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Original contribution

High minichromosome maintenance protein 7 proliferation indices: a powerful predictor of progression in pancreatic neuroendocrine neoplasms without distant metastasis at the time of surgery☆,☆☆

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Summary Pancreatic neuroendocrine neoplasms (PanNENs) have an unpredictable clinical course that varies from indolent to highly malignant. No immunohistochemical markers are available for reliable prediction of the biological behavior of early stage PanNENs. Minichromosome maintenance protein 7 (MCM7) is a putative powerful marker of cell proliferation. Whether the expression of MCM7 is related to the risk of PanNENs progression remains unclear. We assessed the clinical behavior of 156 PanNENs with respect to stage, grade, Ki-67 index, MCM7 index, and other pathologic features. A high MCM7 index was significantly associated with larger tumor size ($P < .001$), nonfunctioning tumor ($P < .001$), increased grade ($P < .0001$), and later TNM stage ($P < .001$). In multivariate analysis, G2/G3 (hazard ratio [HR], 2.21; 95% confidence interval [CI], 1.35-3.62; $P < .001$), stage III/IV (HR, 2.11; 95% CI, 1.31-3.41; $P < .001$), and MCM7 labeling index >5% (HR, 3.81; 95% CI, 1.30-11.17; $P = .02$) were independent negative prognostic factors related to the risk of tumor progression in stage I-IV disease. MCM7 labeling index >5% was associated with an increased risk of progression in stages I-V, I-III, and I-II. Our study confirms that MCM7 is a valuable marker for

Abbreviations: PanNEN, pancreatic neuroendocrine neoplasm; MCM7, minichromosome maintenance protein 7; WHO, World Health Organization; PFS, progression-free survival; PanNEC, pancreatic neuroendocrine carcinoma; PanNET, pancreatic neuroendocrine tumor; PUMCH, Peking Union Medical College Hospital; LI, labeling index; OS, overall survival; HPF, high-power field; H&E, hematoxylin and eosin; MI, mitotic index; TNM, tumor-node-metastasis; HR, hazard ratio; CI, confidence interval; IHC, Immunohistochemistry; ROC, Receiver operating characteristic; SSA, Somatostatin analog.

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assessing the progression of PanNENs, especially in patients with early stage disease and without distant metastasis.

1. Introduction

PanNENs, as defined by the 2017 WHO *Classification of Tumours of Endocrine Organs* [1], include well-differentiated PanNETs, poorly differentiated PanNECs, and mixed neuroendocrine nonneuroendocrine neoplasms.

The incidence and diagnosis of PanNENs (particularly nonfunctioning PanNENs) has steadily increased over the past 40 years [1-6]. In clinical practice, the prognosis of a patient determines the frequency of the follow-up visits. Better evaluation of long-term outcome and progression risk in patients with PanNENs are desirable [7,8]. The tumor stage is naturally predictive of the outcome of these patients; however, it has been difficult to predict which tumors are prone to recurrence and metastasis by pathologic examination alone [9]. Several prognostic markers of PanNENs have been introduced, but many require further validation. Furthermore, among high-grade (G3) PanNENs, distinguishing PanNETs from PanNECs can be challenging. Although the Ki-67 proliferation index has statistical significance for predicting the behavior of PanNENs, issues regarding the proposed Ki-67 cutoffs persist. Furthermore, studies have questioned the accuracy and reproducibility of the Ki-67 index, especially for tumors with equivocal grades; therefore, markers for evaluating clinical behavior, especially early stage PanNENs, are still needed [9,10].

Cell division is a tightly regulated process that involves the contribution of many proteins. Minichromosome maintenance (MCM) proteins are essential for the initiation and elongation of DNA replication and are claimed to be powerful markers of cell proliferation. MCM7 is a member of the MCM protein family that is important for cell cycle progression [11]. MCM7 has been shown to be an important prognostic marker in many malignant neoplasms [12-16], including lung cancer, prostate cancer, progressive cervical disease, oral squamous cell carcinoma, and pituitary adenoma. MCM7 is widely used as a diagnostic and prognostic marker for cancer, and MCM7 is thought to provide a more sensitive readout than Ki-67 for detecting proliferating cells [17]. Whether the expression of MCM7 is related to the risk of PanNEN progression remains unclear; thus, further evaluations and comparisons are warranted.

In the current study, we assessed the expression of MCM7 using IHC staining in comparison with the conventional proliferation marker Ki-67 and the clinical behavior of 156 PanNENs with respect to stage, grade, and other pathologic features.

2. Materials and methods

2.1. Case selection

In a retrospective review of the surgical pathology files at PUMCH, we identified 156 patients with PanNENs who were treated between July 2003 and November 2016 and had samples available for immunohistochemical studies. Hematoxylin and eosin (H&E)–stained slides of the samples were independently reviewed by 3 senior pathologists from PUMCH, according to the WHO 2017 guidelines. Tumors included 150 primary PanNENs and 6 metastases (4 involving the liver, 1 involving the kidney, and 1 involving the lymph node). Among the 150 patients with resected primary PanNETs, 7 had material available from the metastatic site for immunohistochemical analysis; the specimens included 2 lymph node and 5 liver samples.

Clinical information on age, symptoms, presence of metastases at the time of diagnosis, evidence of local invasion at surgery, and evidence of local recurrence or distant metastases during follow-up was collected from patient charts and clinical records or by contacting patients and family members. Follow-up evaluation after resection consisted of physical examinations, laboratory tests, and radiologic imaging. The frequency of hospital visits was at least every 6 months for the first 2 years and yearly thereafter. The duration of follow-up was calculated from the date of surgery to the date of recurrence/progression, death, or last follow-up. Recurrence/progression was defined as local recurrence in the pancreas, a new location in the lymph nodes, or the development of distant metastases. All recurrences/progressions were identified through radiologic imaging. This study was approved by the institutional review board of PUMCH.

In all cases, we reviewed H&E-stained slides that were prepared from routinely processed partial pancreatectomy specimens (including pancreaticoduodenectomy and distal pancreatectomy) and/or resected metastases that were fixed in 10% buffered formalin.

Based on proliferative indices, PanNETs were graded as G1 (<2 mitoses per 10 HPFs and Ki-67 proliferation index <3%), G2 (2-20 mitoses per 10 HPFs or Ki-67 proliferation index of 3%-20%), or G3 (>20 mitoses per 10 HPFs or Ki-67 proliferation index >20%). PanNECs were composed of highly atypical small cells or intermediate-to-large cells, in view of proliferative activity (> 20 mitoses per 10 HPFs or Ki-67 proliferation index >20%).

The stage of PanNETs was determined according to the WHO 2017 TNM classification of tumors of the

neuroendocrine pancreas [1]; the WHO 2017 classification and staging of PanNENs follows the eighth edition American Joint Committee on Cancer/Union for International Cancer Control TNM staging system and corresponds to the European Neuroendocrine Tumor Society TNM classification. The TNM classification of PanNECs follows the ductal adenocarcinoma classification criteria.

2.2. Immunohistochemistry

The IHC protocol was adapted from a study by Intartaglia et al [18]. Tumor tissues were fixed in 10% neutral buffered formalin and embedded in paraffin, according to standard procedures. Four-micrometer-thick slides were deparaffinized with xylene and were rehydrated, and the slides were immersed in a preheated solution of citrate retrieval buffer in an autoclave. Nonspecific antibody reactivity was blocked by incubating the tissue with blocking buffer (prediluted serum, ZLI-9022; ZSGB-BIO, Beijing, China). The tissue sections were incubated with primary antibody overnight at 4°C. The slides were incubated in a 3% hydrogen peroxide solution in methanol for 30 minutes at room temperature, in the dark. Two drops of secondary antibody conjugated to horseradish peroxidase (PV-6002; ZSGB-BIO) were added, and the slides were incubated in a humidified chamber in the dark for 30 minutes. Two drops of 3,3′-diaminobenzidine solution (ZLI-9017; ZSGB-BIO) were added to the tissue section, and the samples were incubated for 3 to 6 minutes, until the signal was observable under the microscope. The following primary antibodies were used: anti-MCM7 (1:200, clone 141.2; Santa Cruz Biotechnology, Dallas, TX) and anti–Ki-67 (prediluted, clone MIB-1; ZSGB-BIO). MCM7 and Ki-67 proteins were practically undetectable in normal islet tissue. Negative controls were prepared by omitting the primary antibodies. The germinal center of the lymph nodes was used as a positive control.

2.3. Evaluation of Ki-67, MCM7, and MIs

Five HPFs with the strongest nuclear labeling, each containing at least 2000 cells, were enumerated independently by 2 pathologists, and the percentage of Ki-67 and MCM7 immunostained nuclei was calculated as the LI. The MIs, reported as the number of mitoses in 10 HPFs, was obtained by counting the number of mitoses in 50 HPFs and dividing that number by 5.

2.4. Statistical analysis

Recurrence/PFS was calculated from the date of the first surgery to the date of recurrence/progression. PFS and OS curves were analyzed using the Kaplan-Meier method, and the log-rank test was used for the assessment of plots between groups. Univariate Cox proportional hazards regression models were fit to determine the association between survival and clinical and pathologic characteristics. Significant factors in the univariate models were included in a multivariate Cox proportional hazards regression model, to assess the association between survival and clinical and pathologic characteristics, upon adjusting for other factors. ROC analysis was performed to investigate the diagnostic ability with regard to PFS and OS. *P* values of <.05 were deemed to be significant,

Table 1 Clinicopathological characteristics of 156 patients with PanNENs

Characteristics	No. (%)
Age at surgery (y), median (range)	48 (13-72)
Sex	
Female	42 (26.9)
Male	114 (73.1)
Associated syndrome	
MEN1	11 (7.1)
Nonfunctioning	66 (42.3)
Functioning	90 (57.7)
Insulinoma	76 (48.7)
Noninsulinoma functioning	14 (9.0)
Primary tumor location	
Head and/or neck	48 (30.8)
Body and/or tail	108 (69.2)
Tumor size (cm), median (range)	2 (0.6-9)
Metastasis	
No metastasis	109 (69.9)
LN metastasis only	21 (13.5)
Liver metastasis only	12 (7.7)
LN and liver metastasis	10 (6.4)
Other	4 (2.5)
WHO grade	
Well-differentiated PanNENs: PanNETs	
Pan NET G1	77 (49.4)
Pan NET G2	70 (44.9)
Pan NET G3	2 (1.3)
Poorly differentiated PanNENs: PanNECs	
PanNEC G3	7 (4.4)
TNM stage	
I	57 (36.5)
II	54 (34.6)
III	23 (14.7)
IV	22 (14.1)
Follow-up information	
Follow-up months, median (range)	53 (5-185)
Not available	5 (3.2)
Available	151 (96.8)
Alive without disease	107 (68.6)
Alive with disease	29 (18.6)
Died of neoplasm	15 (9.6)
Postoperative adjuvant therapies information	
SSAs	18 (11.5)
SSAs/targeted therapies	6 (3.9)
Cytotoxic therapies	9 (5.8)
Local therapies	5 (3.2)
No specific therapies	113 (72.4)
Not available	5 (3.2)

and statistical analyses were performed using IBM SPSS 21 software (IBM, Armonk, NY).

3. Results

3.1. Clinical and pathologic features

The clinical and pathologic features of the 156 patients with PanNENs are summarized in Table 1. The median age of patients was 48 years (range, 13-72 years), and 114 (73.1%) were men. Eleven (7.1%) patients had multiple endocrine neoplasia type 1. Ninety (57.7%) PanNENs were functional, including 76 insulinomas, 5 gastrinomas, 6 vipomas, and 3 unknown types. Sixty-six patients had nonfunctioning tumors.

According to the WHO 2017 classification standards, 49.4% (77 cases) of the tumors were G1 PanNETs, 44.9% (70 cases) were G2 PanNETs, 1.3% (2 cases) were G3 PanNETs, 2.5% (4 cases) were small cell–type G3 PanNECs, and 1.9% (3 cases) were large cell–type G3 PanNECs. Based on the WHO 2017 staging manual, 57 patients (36.5%) had stage I disease, 54 (34.6%) had stage II disease, 23 (14.7%) had stage III disease, and 22 (14.1%) had stage IV disease.

After surgery, patients participated in a follow-up program and received appropriate therapy in case of recurrence/progression. During the follow-up period (median, 53 months; range: 5-185 months), 45 (28.8%) patients had recurrence/progression. Eighteen (11.5%) patients used SSA therapies, 6 (3.8%) used SSAs and targeted therapies, 9 (5.8%) patients used cytotoxic therapies, 5 (3.2%) used local therapies, and 113 (72.4%) patients did not use any specific therapies. The treatment information of 5 (3.2%) patients was unavailable.

3.2. Distribution of Ki-67 and MCM7 LIs and MIs

We performed MCM7 and Ki-67 IHC using whole-tissue sections from the 156 patients with resected PanNENs. MCM7 nuclear staining was absent in the islets of Langerhans in the adjacent normal pancreatic tissue and was rarely found in the pancreatic acinus. Fig. 1 shows representative images

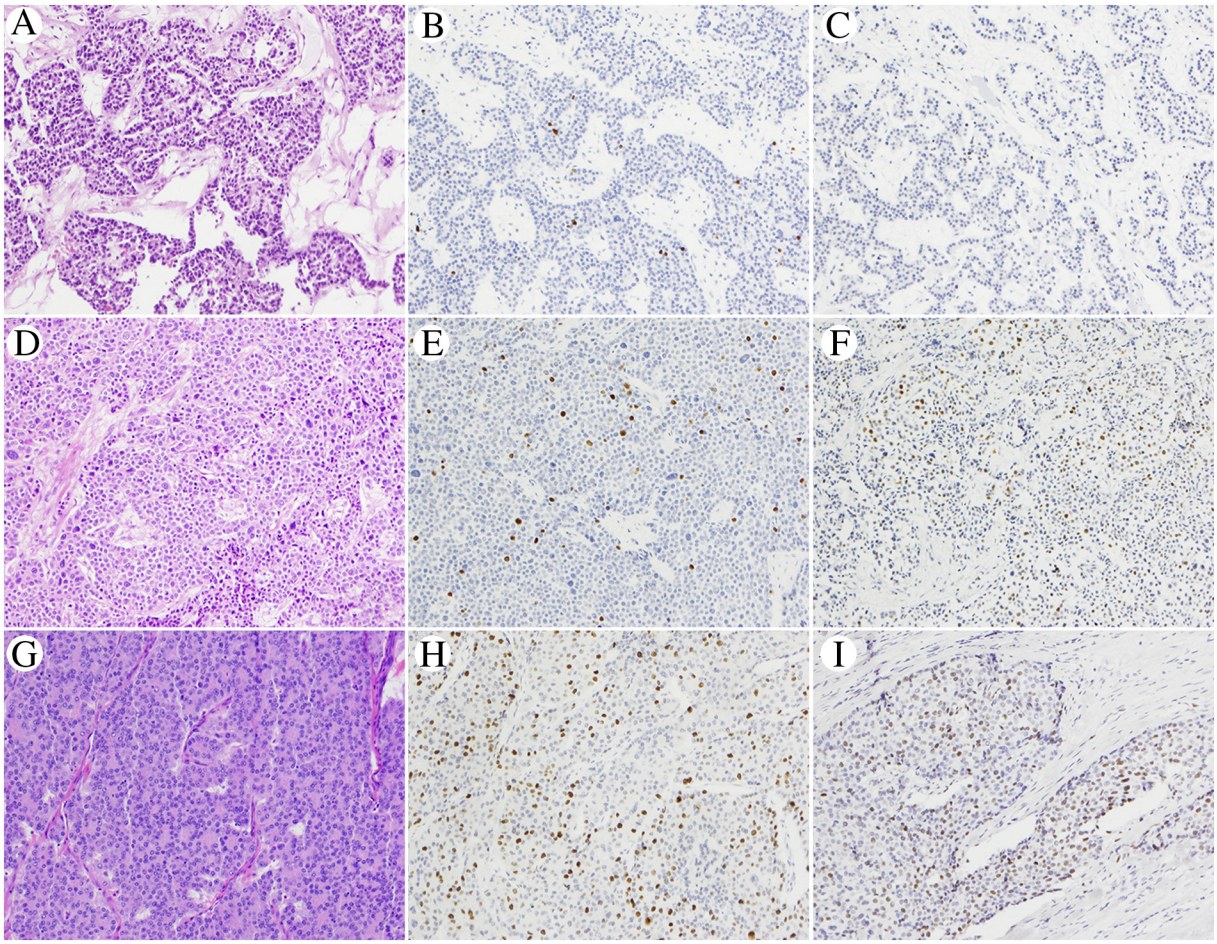

Fig. 1 Representative images of H&E staining (A, D, and G), Ki-67 (B, E, and H), and MCM7 (C, F, and I) immunolabeling in PanNEN tissues. The percentage of cells staining positive for MCM7 and Ki-67 increased with increasing tumor grade and stage. A-C, A case of PanNEN G1/stage I: both MCM7 and Ki-67 LIs were <5%. D-F, Example of PanNEN G2/stage III showing Ki-67 and MCM7 LI 5% and 10%, respectively. G-I, A case of PanNEN G3/stage IV, suggesting Ki-67 and MCM7 LI > 20% (original magnification ×100 [A-I]).

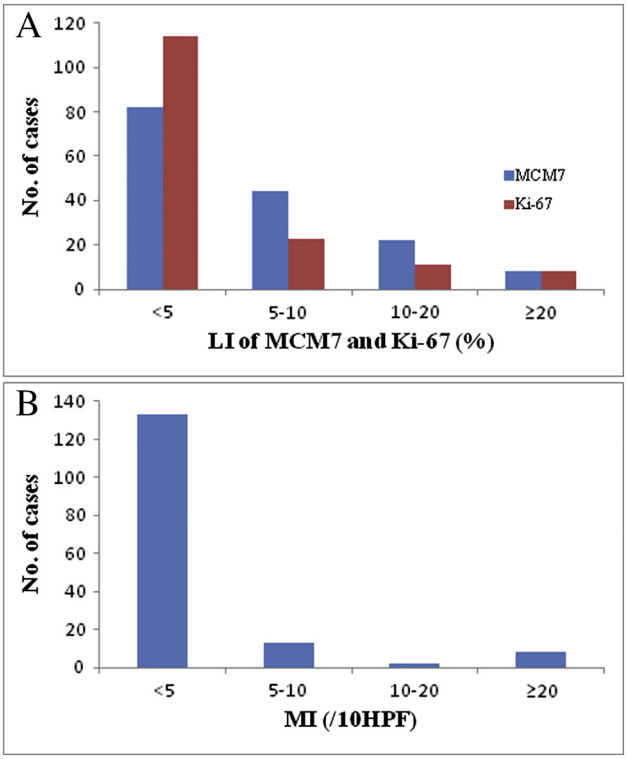

Fig. 2 Distribution of Ki-67 and MCM7 LIs and MIs in 156 patients with PanNEN. A, Ki-67 and MCM7 LI distribution. B, MI distribution.

of H&E staining and Ki-67 and MCM7 immunolabeling in PanNEN tissues according to the indicated grades. The percentage of cells staining positive for MCM7 and Ki-67 increased with increasing tumor grade and stage.

The distribution of Ki-67 and MCM7 LIs and MIs in the 156 PanNENs is illustrated in Fig. 2. Most cases had either 0% or very low LIs and MIs for MCM7 and Ki-67. A total of 44 (28.2%) cases had MCM7 LIs ranging from 5% to 10%, and 22 (14.1%) cases displayed MCM7 LIs of 10% to 20%; very few cases (8; 5.1%) had LIs greater than 20% (Fig. 2A). A total 23 (14.7%) cases had Ki-67 LIs ranging from 5% to 10%, 11 (7.1%) cases displayed Ki-67 LIs of 10% to 20%, and very few cases (8; 5.1%) were greater than 20% (Fig. 2A). For MIs, 13 (8.3%) cases ranged from 5% to 10%, 2 (1.3%) cases displayed MIs of 10% to 20%, and very few cases (5.1%) had MIs greater than 20% (Fig. 2B).

There were a greater number of cases with MCM7 LIs of 5% to 10% (28.2%) than cases with Ki-67 LIs 5% to 10% (14.7%).

3.3. MCM7, Ki-67 staining, MIs, and clinicopathological features

The percentage of cells staining positive for MCM7 increased with increasing tumor size ($P < .001$), grade ($P < .001$), and TNM stage ($P < .001$; Table 2). The LIs of Ki-67 and MI also increased significantly with increasing age, tumor

Table 2 MCM7, Ki-67, and MI according to clinicopathological characteristics of PanNENs patients

Characteristics	n	MCM7 LI (%)	P[a]	Ki-67 LI (%)	P	MI (/10 HPFs)	P
Age at surgery ())							
<50	86	3.7 ± 0.3[b]		2.6 ± 0.2		1.8 ± 0.2	
≥50	70	11.1 ± 2.0	<.001	9.5 ± 2.2	.002	4.9 ± 1.0	.001
Size (cm)							
<2	60	3.7 ± 0.8		2.2 ± 0.5		1.6 ± 0.3	
2-4	53	6.4 ± 1.1		5.4 ± 1.2		3.6 ± 0.7	
≥4	43	12.3 ± 2.8	<.001	10.9 ± 3.1	<.001	5.0 ± 1.3	<.001
Functioning							
No	66	11.4 ± 2.1		10.0 ± 2.3		5.2 ± 1.0	
Yes	90	3.7 ± 0.3	<.001	2.6 ± 0.3	<.001	1.7 ± 0.3	<.001
Functioning							
Insulinoma	76	3.0 ± 0.2		1.9 ± 0.1		1.3 ± 0.1	
Noninsulinoma	14	8.0 ± 1.4	<.001	6.1 ± 1.3	<.001	3.9 ± 1.5	<.001
Grade							
G1	77	2.5 ± 0.2		1.3 ± 0.1		1.0 ± 0.0	
G2	70	6.7 ± 0.5		5.1 ± 0.4		3.1 ± 0.2	
G3	9	47.4 ± 7.3	<.001	48.3 ± 9.2	<.001	22.7 ± 3.9	<.001
Stage							
I	57	2.9 ± 0.2		1.8 ± 0.2		1.2 ± 0.1	
II	54	5.0 ± 1.0		4.3 ± 1.2		2.7 ± 0.6	
III	23	13.1 ± 3.5		12.4 ± 4.8		5.6 ± 1.4	
IV	22	16.0 ± 4.3	<.001	12.5 ± 3.6	<.001	7.0 ± 2.4	<.001

[a] Mann-Whitney test.
[b] Mean ± SEM.

size, and TNM stage ($P < .001$; Table 2). The percentage of cells staining positive for MCM7, Ki-67, and MI in nonfunctioning PanNENs and noninsulinomas was greater than in insulinomas ($P < .001$; Table 2).

3.4. PFS in patients with stage I-IV disease

For analysis of recurrence/PFS, MCM7 and Ki-67 LI values were converted to binomial variables, based on ROC analysis. According to the ROC analysis, cutoff values for MCM7 and Ki-67 were >5% and ≥5%, respectively. PFS curves for patients with high or low MCM7 and Ki-67 LIs are shown in Fig. 3. Kaplan-Meier analysis of PFS curves revealed that patients with high MCM7 LIs (>5%) as well as high Ki-67 LIs (≥ 5%) had shorter PFS for stage I-IV, stage I-III, and stage I-II disease than did patients with low MCM7 LIs (≤5%) and low Ki-67 LIs (<5%; log-rank test, $P < .001$).

Various associations of the features studied with PFS are summarized in Table 3. Increasing age and tumor size, higher grade, noninsulinoma, stage T3/T4, lymph node metastasis, stage III/IV, MCM7 LI > 5%, and Ki-67 LI ≥ 5% were associated with increased risk of tumor progression after surgery, in univariate analysis. As revealed in multivariate analysis, G2/G3 (HR, 2.21; 95% CI, 1.35-3.62; $P < .001$), stage III/IV (HR, 2.11; 95% CI, 1.31-3.41; $P < .001$), and MCM7 LI> 5% (HR, 3.81; 95% CI, 1.30-11.17; $P = .02$) retained independent negative prognostic significance related to the risk of tumor progression.

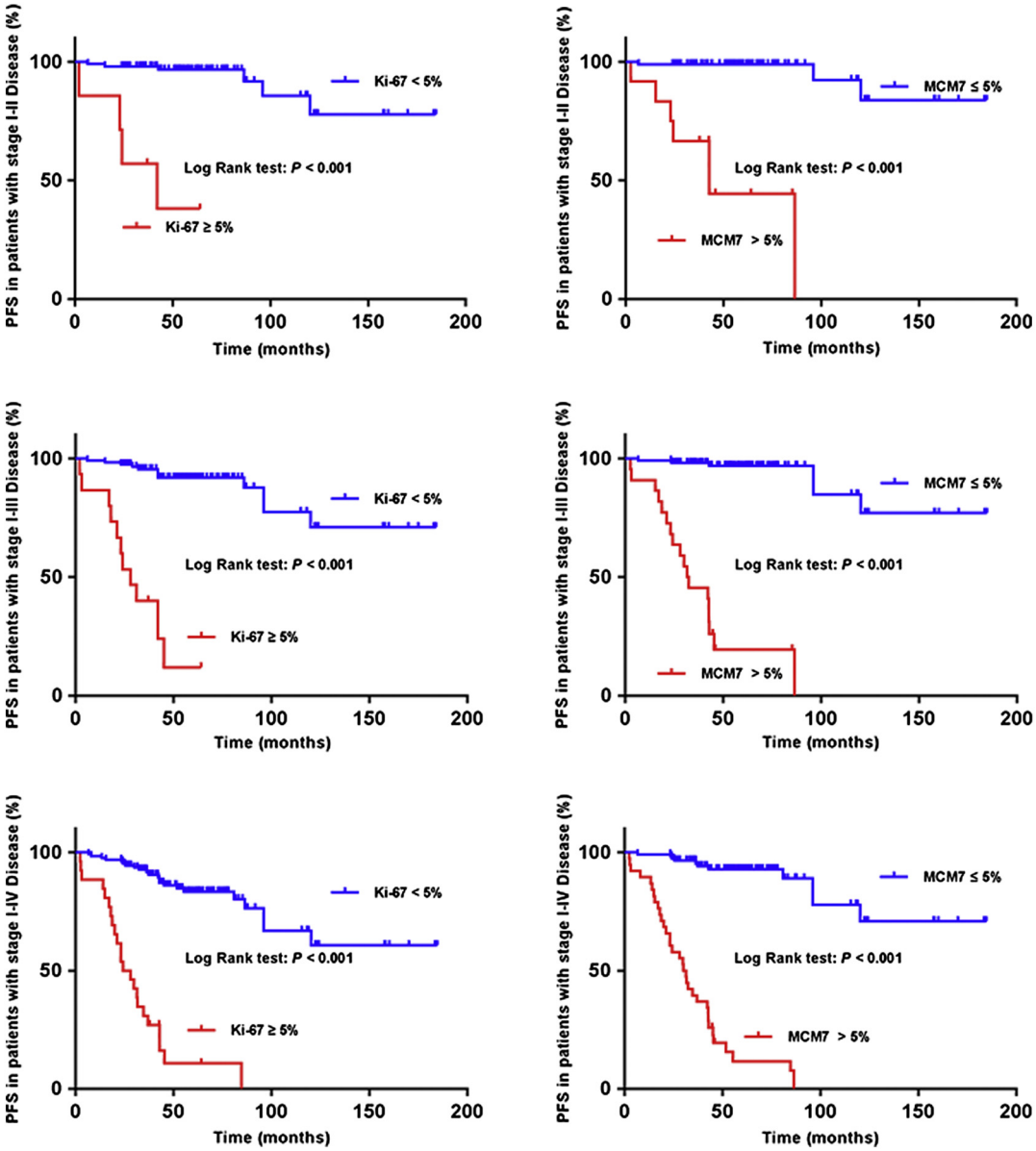

Fig. 3 Kaplan-Meier survival curves according to MCM7 LI and Ki-67 LI. Patients with higher MCM7 LI or Ki-67 LI showed a significant tendency toward progression. The *x*-axis corresponds to follow-up in months, and the *y*-axis corresponds to PFS.

Table 3 Univariate and multivariate analyses of PFS in stage I-IV PanNENs patients

Characteristics	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P* [a]	HR (95% CI)	*P*
Age	1.05 (1.02-1.07)	<.001	–	–
Tumor size	1.37 (1.22-1.63)	<.001	0.85 (0.72-1.00)	.06
Tumor location				
Head	Reference	Reference		
Body/Tail	0.94 (0.51-1.75)	.85	–	–
Insulinoma	Reference	Reference		
Noninsulinoma	11.99 (4.89-29.41)	<.001	2.73 (0.83-8.92)	.1
T stage				
T1/T2	Reference	Reference		
T3/T4	5.70 (3.09-10.52)	<.001	–	–
Positive lymph nodes	8.49 (4.55-15.83)	<.001	–	–
WHO grade				
G1	Reference	Reference		
G2/G3	4.34 (3.14-5.99)	<.001	2.21 (1.35-3.62)	<.001
TNM stage				
I/II	Reference	Reference		
III/IV	3.87 (2.80-5.34)	<.001	2.11 (1.31-3.41)	<.001
MCM7 LI (%)				
≤5	Reference	Reference		
>5	5.36 (3.74-7.68)	<.001	3.81 (1.30-11.17)	.02
Ki-67 (%)				
<5	Reference	Reference		
≥5	3.84 (2.88-5.11)	<.001	1.50 (0.67-3.36)	.33

[a] Likelihood ratio test.

Table 4 Univariate and multivariate analyses of PFS in stage I-III PanNENs patients

Characteristics	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P* [a]	HR (95% CI)	*P*
Age	1.06 (1.03-1.10)	.001	–	–
Tumor size	1.45 (1.23-1.71)	<.001	1.13 (0.89-1.43)	.31
Tumor location				
Head	Reference	Reference		
Body/tail	0.84 (0.37-1.92)	.677	–	–
Insulinoma				
Noninsulinoma	8.25 (2.92-23.30)	<.001	3.05 (0.67-13.87)	.15
T				
T1/T2	Reference	Reference		
T3/T4	7.72 (3.28-18.17)	<.001	–	–
Positive lymph nodes	11.65 (4.95-27.39)	<.001	–	–
WHO grade				
G1	Reference	Reference		
G2/G3	6.36 (3.75-10.77)	<.001	1.04 (0.40-2.74)	.93
MCM7 LI (%)				
≤5	Reference	Reference		
>5	3.84 (2.89-5.11)	<.001	5.29 (2.07-13.51)	<.001
Ki-67 (%)				
<5	Reference	Reference		
≥5	5.34 (3.42-8.35)	<.001	1.11 (0.49-2.51)	.80

[a] Likelihood ratio test.

Table 5 Univariate and multivariate analyses of PFS in stage I-II PanNENs patients

Characteristics	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P [a]	HR (95% CI)	P
Age	1.04 (0.99-1.09)	.125	–	–
Tumor size	1.42 (1.11-1.82)	.005	1.09 (0.75-1.59)	.661
Tumor location				
Head	Reference	Reference		
Body/tail	0.76 (0.21-2.70)	.67	–	–
Insulinoma	Reference	Reference		
Noninsulinoma	7.59 (1.84-31.36)	.005	7.73 (0.76-78.92)	.085
T stage				
T1/T2	Reference	Reference		
T3/T4	5.05 (1.19-21.43)	.028	–	–
WHO grade				
G1	Reference	Reference		
G2/G3	5.01 (2.37-10.59)	<.001	0.28 (0.03-2.31)	.234
MCM7 LI (%)				
≤5	Reference	Reference		
>5	6.64 (3.10-14.23)	<.001	9.88 (2.21-44.11)	.003
Ki-67 (%)				
<5	Reference	Reference		
≥5	4.35 (2.33-8.12)	<.001	1.29 (0.30-5.57)	.733

[a] Likelihood ratio test.

3.5. PFS in patients with stage I-III disease

The features that were significantly associated with PFS in patients with stage I-III disease on univariate analysis included age, tumor size, noninsulinoma, grade, lymph node metastasis, MCM7 LI > 5%, and Ki-67 LI ≥ 5%. On multivariate analysis, MCM7 LI > 5% (HR, 5.29; 95% CI, 2.07-13.51; $P < .001$) was an independent negative prognostic factor with respect to the risk of tumor progression (Table 4).

3.6. PFS in patients with stage I-II disease

Univariate analysis revealed that larger tumor size, noninsulinoma, higher grade, MCM7 LI > 5%, and Ki-67 LI ≥ 5% were associated with an increased risk of progression; as revealed in multivariate analysis, MCM7 LI > 5% (HR, 9.88; 95% CI, 2.21-44.11; $P = .003$) retained independent negative prognostic significance related to the risk of progression (Table 5).

3.7. Overall survival

The median length of follow-up was 53 months and ranged from 5 to 185 months. A total of 107 patients (68.6%) remained alive without evidence of progression at the last follow-up, 29 patients (18.6%) were alive with metastatic disease or progression, and 15 patients (9.6%) died of the disease. The median OS of all patients was not reached. The estimated 3-, 5-, and 10-year survival rates were 94%, 91%, and 78%, respectively. We did not conduct univariate and multivariate analyses related to OS owing to insufficient events of death.

4. Discussion

PanNENs have varying behaviors, ranging from indolent to aggressive. Despite widespread increase in the occurrence of PanNENs, the molecular events underlying disease progression remain unclear. Patterns of gene expression can serve to distinguish aggressive metastatic tumors from more indolent PanNENs, but in the absence of an established genetic signature, the disease is still defined on the basis of clinical criteria. Tumor WHO grades and stages have emerged as the most important factors in predicting aggressive behavior [1,8,19-21]. Our findings confirm the prognostic value of the 2017 WHO grading criteria and TNM staging system for PanNENs. In our study, stage III/IV and higher grade were significantly associated with tumor progression in patients with stage I-IV disease. However, marked differences in survival were observed among patients, even those with the same pathologic grade and stage.

There is a great need for new prognostic markers that can better assess survival probabilities and optimize postoperative management. MCM7 and several other components bind to genome replication origins and allow the DNA polymerase complex to replicate DNA. The expression of these elements is tightly controlled by several signaling pathways, to ensure proper DNA replication. MCM7 is a key component of

prereplication complex assembly at the replication origin during the early G1 phase [22], and MCM7 expression is shut down during the S, G2, and early M phases to prevent the reinitiation of DNA synthesis. MCM7 amplification may reduce the phase-specific control of its expression, and the continuous presence of MCM7 throughout the cell cycle not only would recruit more cells into the proliferation cycle but also could induce the reinitiation of DNA synthesis, thereby increasing the risk of chromosomal abnormalities.

The MCM protein family is involved in numerous essential steps in DNA replication. The requirement for MCM proteins in cycling cells and their absence in quiescent ones provide evidence for their potential clinical application as cell proliferation markers [23]. Abnormally high expression of MCM7 has been detected in several different types of cancer [24-26], and high MCM7 protein expression levels have been demonstrated to correlate with prostate cancer progression [13], poor prognosis of patients with non–small cell lung cancer [27], and the recurrence of colorectal cancer. Using an array analysis technique, Ren et al [13] identified that the MCM7 gene was dramatically amplified in aggressive prostate cancer. Furthermore, Choy et al [28] demonstrated that the percentages of MCM4 and MCM7 expression were significantly correlated with Ki-67, Bmi1, and cyclin E expression in esophageal carcinoma and precancerous lesions. MCM4 and MCM7 may serve as sensitive proliferation markers for the evaluation of esophageal carcinoma and precancerous lesions. MCM2 and MCM3 are also proliferative markers in gastroenteropancreatic neuroendocrine neoplasms, and MCM protein expression is elevated in SI (small intestinal) NENs, relative to the normal mucosa [29].

Here, we examined MCM7 expression in PanNENs and evaluated its potential role as a proliferative and prognostic marker. Normal islets of Langerhans and pancreatic acinar cells seldom exhibited MCM7 positivity, which confirms that few cells have the capacity for replication and most are terminally differentiated. We showed that the presence of high MCM7 indices may enhance our ability to predict PFS in patients with PanNENs, independent of WHO grade and TNM stage. High MCM7 LI showed independent negative prognostic significance, as confirmed by multivariate Cox regression analysis. The strong association of MCM7 protein expression with PanNEN progression may provide clues about the potential role of dysfunctional DNA replication in determining tumor indolence or aggressiveness. Higher MCM7 LI was usually correlated with older age, larger tumor size, higher grade, and later tumor stage in patients with PanNENs. Moreover, we found that MCM7 LIs were associated with patient functional status, and MCM7 was expressed at high levels in patients with nonfunctioning PanNENs and noninsulinoma in functioning PanNENs. Regarding survival, Kaplan-Meier curves showed that patients with high MCM7 LIs had shorter PFS durations in comparison with patients who had low MCM7 LIs. On multivariate analysis, MCM7 LI > 5% was associated with increased risk of progression in stage I-IV, stage I-III, stage I-II disease. We confirmed that MCM7 LI > 5% was an independent risk factor for progression in patients with PanNENs, especially those without distant metastasis at the time of surgery.

There were more cases with MCM7 LI 5%-10% than cases with Ki-67 LI 5%-10%, suggesting that MCM7 could identify more patients with high risk of progression than Ki-67. We believe that MCM7 offers great advantages over other proliferation biomarkers, as MCM7 is not expressed in cells undergoing DNA repair [30,31] and can identify abnormal precursor cells with higher sensitivity than Ki-67. MCM7 might be an independent prognostic marker that is more reliable than Ki-67 in early stage PanNENs. MCM7 could therefore serve as a good indicator for predicting the prognosis of patients with PanNENs, to allow for more intensive follow-up and appropriate therapy in cases of recurrence/progression via determining the MCM7 expression status of resected specimens.

In our study, we did not attempt to refute the value of the Ki-67 index for predicting the clinical behavior of PanNENs. Genc et al [32] confirmed that patients at high risk of recurrence after curative resection of G1 or G2 PanNET can be identified using a Ki-67 LI higher than 5%. We confirmed that Ki-67 LI $\geq$ 5% was associated with increased risk of tumor progression after surgery, according to univariate survival analysis. Although multivariate survival analysis did not yield statistical significance, Ki-67 was still a valuable marker in determining the proliferation potential and progression of PanNENs.

The identification of survival-associated factors paves the way for further exploration of the mechanistic basis of disease and provides opportunities for better care of patients with PanNENs. However, the discovery itself and the initial validation only represent the first steps on a long journey toward routine clinical implementation. In conclusion, we confirmed that MCM7 is a valuable marker for assessing the progression of PanNENs, especially in patients with early stage disease and without metastasis; however, our results require further long-term validation. We suggest that patients with MCM7 LI > 5% should be more closely monitored postoperatively to detect progression earlier and that these patients might benefit from adjuvant treatment. External validation with a different cohort is needed to investigate whether the cutoff for MCM7 LI as a predictor of progression risk is valid for worldwide use.

CRediT authorship contribution statement

Xinchao Ban: Data curation, Formal analysis, Methodology, Writing - original draft. **Jie Yan:** Resources, Formal analysis, Investigation. **Shuangni Yu:** Project administration, Writing - review & editing. **Zhaohui Lu:** Project administration, Validation, Visualization. **Xiaoyan Chang:** Project

administration, Validation, Visualization. **Congwei Jia:** Formal analysis, Resources. **Cen Gao:** Software, Data curation. **Huilin Shao:** Software, Visualization. **Yan Wu:** Software, Visualization. **Xinxin Mao:** Formal analysis, Resources. **Yue Zhang:** Software, Data curation. **Yuan Li:** Project administration, Validation, Visualization. **Jie Chen:** Conceptualization, Funding acquisition, Supervision.

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Author contributions

Jie Chen: experimental design, supervision, and results analysis

Xinchao Ban: experiments, manuscript writing, data analysis

Jie Yan: sample collection and data analysis

Shuangni Yu: critical reading and revision of the manuscript

Zhaohui Lu, Xiaoyan Chang, and Yuan Li: H&E slide review and diagnosis

Congwei Jia and Xinxin Mao: evaluation of IHC staining for Ki-67, MCM7, and MI.

Huilin Shao and Yan Wu: figure and table construction

Cen Gao and Yue Zhang: acquisition of clinical information.

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