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Ki-67 and presence of liver metastases identify different progression-risk classes in pancreatic neuroendocrine neoplasms (pNEN) undergoing resection

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ABSTRACT

In pancreatic neuroendocrine neoplasms (pNEN), size ≤2 cm and Ki-67 < 3% suggest indolent behavior, but no factor alone predicts prognosis. We investigated factors predictive of tumor progression in 80 pNENs surgically resected in a single Institution from 1995 to 2015. At multivariable analysis the only two independent variables related to PFS were Ki-67 (HR 2.97; 95%CI 1.26–7.02) and presence of synchronous liver metastases (HR 3.60; 95%CI 1.70–7.61). Using Ki-67 < 3% and M0 as reference, the HR for tumor progression was 3.21 (95%CI 1.18–8.74) for M0 patients with Ki-67 3–20%, 5.06 (2.29–11.2) for M1 patients with Ki-67 ≤ 20% and 24.3 (6.64–89.2) for those with Ki-67 > 20%. Tumor size (≤2 vs. >2 cm) was not a predictive factor at any analysis. Intra-class correlation of Ki-67 values on pre-surgical biopsies vs. surgical specimens was 0.99 and Ki-67 classes were correctly identified in 97% of biopsies.

Ki-67 and presence of liver metastases are the major prognostic factors in pNEN and identify different progression risks regardless of tumor size. Pre-surgical pNEN biopsy for Ki-67 assessment should be included in the evaluation of patients with 1–2 cm tumors to help in the decision on whether to perform surgical resection.

Introduction

Pancreatic well-differentiated non-functioning neuroendocrine neoplasms (pNEN) represent a heterogeneous class of tumors, whose clinical behavior mostly relies on the clinico-pathological characteristics of tumor differentiation, Ki-67 labeling index (Ki-67) and occurrence of distant metastases [1,2]. Previous studies established that Ki-67 represents a prognostic biomarker in surgically resected gastro-entero-pancreatic neoplasms (GEP-NET) including pNENs [1,3]. Incremental values of Ki-67 identify different risk categories of patients subjected to surgery [2,4,5]. The current guidelines suggest that surgical intervention is mandatory in early pNEN >2 cm or in smaller (≤2 cm) tumors with a yearly >0.5 cm size increase [6]. On the other hand, patients with ≤2 cm tumors which are clinically stable and do not show any sign of distant metastases are usually treated conservatively within observational protocols [7,8]. In contrast, retrospective analyses report that up to 6% of cases of small pNEN do show unfavorable morphological features and high Ki-67 when subjected to surgery [1,9].

In a consecutive series of pNEN patients undergoing partial pancreatectomy, we studied whether incremental values of Ki-67

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labeling index could be an independent predictor of tumor progression, irrespective of the tumor size. Focusing on a complete pathological characterization of pre- and post-surgical biopsies, we aimed at possible re-definition of the current approach to treatment of pNEN, particularly when tumor size is still around 2 cm and surgical/interventional options are available.

Material and methods

A prospectively maintained clinical database of a single referral center for neuroendocrine neoplasms was interrogated. All consecutive patients with a histological diagnosis of pNEN who underwent surgical resection over the period 1995–2015 were considered. Patients with lung NETs or other non-GEP sites were excluded.

Overall, 80 patients were identified, 35 (43.8%) of which had synchronous liver metastases. The tumor size was ≤2 cm in 38 patients (47.5%) and >2 cm in 42 (52.5%) patients. In 35 cases (43.8%), pre-surgical biopsies (made through endoscopic ultrasound-guided fine needle (19–25 G) aspiration (EUS-FNA) were available and were matched with post-surgical pathology. For the purpose of the study, all specimens and slides were independently and blindly reviewed by three experienced pathologists (MM, PS, AP) using a multi-headed microscope in order to confirm the neuroendocrine nature of the tumor and to perform the morphological evaluation. The occurrence of neuroendocrine differentiation was assessed by immunohistochemistry with anti-synaptophysin and chromogranin-A antibodies. According to WHO guidelines, Ki-67 was evaluated using the MIB1 antibody in at least 2000 cells within the "hot-spot" tumor areas. Manual and automated Ki-67 readings of all specimens, including the 35 diagnostic pre-surgical biopsies, were compared through the automated canScope XT (Aperio, Vista, CA, USA), by scanning slides in the same areas at 40 and evaluating the digital pictures with the Aperio Imagescope Software (Aperio automated computer-assisted quantitative method), or by taking a digital image of the same areas and manually counting the Ki67 positive and negative nuclei on the screen using ImageJ Software (NIH, Bethesda, MA, USA; http://rsbweb.nih.gov/ij/) (computer-assisted manual count) [10]. In all cases, the mitotic index (MI) in 50 HPF (1 HPF = 2 mm^2) was determined. According to the WHO 2017 WHO guidelines, aggregate data of MI and Ki-67 were used to classify pNENs [11]. After pancreatic resection, patients were followed up in a dedicated outpatient clinic. According to ENETS guidelines, physical exams, laboratory tests, tumor markers (chromogranin A) and radiologic imaging were performed at least every 6 months for the first 3 years and yearly thereafter. CT scan was routinely used during follow-up, while MRI scan and nuclear medicine scans (octreoscan and ^{68}Gallium-DOTA-DOC PET) were performed as indicated. Recurrences were identified and censored through radiologic imaging and biopsies when appropriate. The study was conducted according to the clinical standards of the Declaration of Helsinki. All data were anonymized. The observational nature of the study and its methodology were approved by the Institutional Review Board.

Statistical analysis

The association between pathological features and size of primary tumor (≤2 cm or >2 cm) were assessed using the Fisher exact test, while association between pathological features Ki-67 (<3%, 3–20%, >20%) was assessed using the Mantel Haenszel test for trend.

Progression-free survival (PFS) and overall survival (OS) were defined as the time from surgical tumor removal to disease progression or death for any cause, whichever occurred first. PFS and OS curves were drawn using the Kaplan–Meier method. The log-rank test was used to assess the survival difference between patients' groups. Univariable and multivariable Cox proportional regression analysis were used to assess the association between clinico-pathological characteristics and disease progression or death. The variables considered for the analysis included tumor location, tumor size (>2 cm vs. ≤2 cm), Ki-67 (<3%, 3–20%, >20%), mitotic index (MI), multifocality, pancreatic infiltration, peritumoral vascular and/or perineural invasion, tumor necrosis, lymph nodal status, liver metastases and tumor stage according to ENETS AJCC [12,13]. Concordance of pre- and post-operative Ki-67 determination, as well as inter-observer variations among pathologists, were assessed by the weighted Kappa statistics (using Ki-67 as a categorical variable, <3%, 3–20%, >20%) and by the intra-class correlation coefficient (ICC) (using Ki-67 as a continuous variable). Agreement was evaluated considering readings from all the available samples and separately from biopsies and post-surgical samples. Data analysis was performed using the SAS software (version 9.4, Cary NC, USA). All tests were two-sided and p-values <0.05 were considered statistically significant.

Results

Clinico-pathological characteristics, including stratification based on Ki-67 values, and main survival of the different categories are shown in Table 1 and Fig. 1.

Table 1
Clinical and pathologic features of 80 patients who underwent resection of pNEN.

	All	Size of primary tumor (cm)			Ki-67 in primary pancreatic tumors			
		≤2 cm	>2 cm	P	<3%	3–20%	>20%	P
	N	N	N	Exact	N	N	N	trend
All	80	38	42		46	30	4	
Location								
Head	25	10	15		14	10	1	
Body/tail	55	28	27	0.47	32	20	3	0.83
Multiple neoplasms								
Absent	72	33	39		40	28	4	
Present	8	5	3	0.47	6	2	0	0.39
Primary tumor mitotic index								
<2	34	20	14		30	4	–	
≥2	46	18	28	0.11	16	26	4	0.008
Acinic pancreas infiltration								
Absent	26	20	6		21	5	–	
Present	54	18	36	0.0003	25	25	4	0.05
Vascular invasion								
Absent	39	25	14		28	10	1	
Present	41	13	28	0.007	18	20	3	0.15
Perineural infiltration								
Absent	52	29	23		37	13	2	
Present	28	9	19	0.06	9	17	2	0.18
Necrosis of primary tumor								
Absent	71	37	34		44	24	3	
Present	9	1	8	0.03	2	6	1	0.19
Lymph node metastases								
Absent	53	31	22		35	17	1	
Present	27	7	20	0.009	11	13	3	0.03
Synchronous liver mets								
Absent	45	26	19		30	15	–	
Present	35	12	23	0.04	16	15	4	0.01
Stage ENETS/AJCC								
I	22	22	–		17	5	–	
IIA	6	–	6		4	2	–	
IIB	2	–	2		1	1	–	
IIA	3	–	3		2	1	–	
IIIB	33	11	22		12	17	4	
IV	14	5	9	<.0001	10	4	–	0.11

P Exact: P-value based on the Fisher Exact test; P Trend: P-value based on the Mantel Haenszel test for trend.

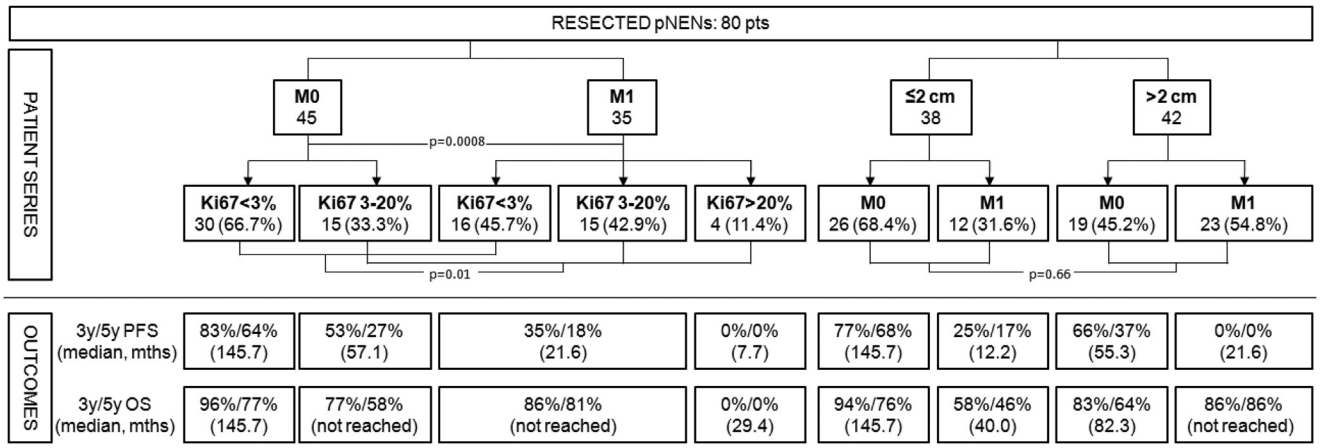


Fig. 1. Distribution of Ki67, primary tumor size, presence of liver metastases and main outcome of 80 consecutive patients with pNENs who underwent pancreatic resection. M1 patients had a significantly lower PFS as compared to M0 patients (multivariable p = 0.0008); patients with Ki67 < 3% had significantly better PFS than the ones with Ki67 ≥ 3% (multivariable p = 0.01); there were no significant differences in PFS according to tumor size ≤2 cm or >2 cm (multivariable p = 0.66).

Forty-five patients (56.3%) were free of metastases (M0) and 35 (43.8%) were M1. Out of the 45 M0 patients, 30 (66.7%) showed a Ki-67 < 3% and 15 (33.3%) between 3% and 20%. Out of the 35 M1 patients, 16 (45.7%) had Ki-67 < 3%, 15 (42.9%) between 3% and 20% and 4 had Ki-67 > 20%. The median tumor size was 25 mm (range 4–150), with 38 cases ≤2 cm (median size: 12 mm). Synchronous metastases were observed in 12/38 (31.6%) of patients with tumors ≤2 cm and in 23/42 (54.8%) patients with tumors >2 cm.

Table 2 and Fig. 2 show the results of the univariable and multivariable survival analysis [13,14]. Median follow-up of the whole series was 45 months and median PFS was 50.5 months (95% CI 24.7–57.6). Median OS was not reached, with 61 patients (76.3%) still alive at the end of the observation period. At univariable analysis, the occurrence of synchronous liver metastases (HR 3.63; 95%CI 1.98–6.64), lymph node spread (HR 1.90; 95%CI 1.07–3.36), pancreatic infiltration (HR 2.23; 95%CI 1.11–4.49), perineural invasion (HR 1.98; 95%CI 1.11–3.53) and Ki-67 ≥ 3% (HR 2.57; 95%CI 1.43–4.61) were significantly associated with tumor progression. Multivariable analysis showed that the only two independent variables related to PFS were Ki-67 ≥ 3% (HR 2.97; 95%CI 1.26–7.02) and the occurrence of liver metastases at diagnosis (HR 3.60; 95%CI 1.70–7.61).

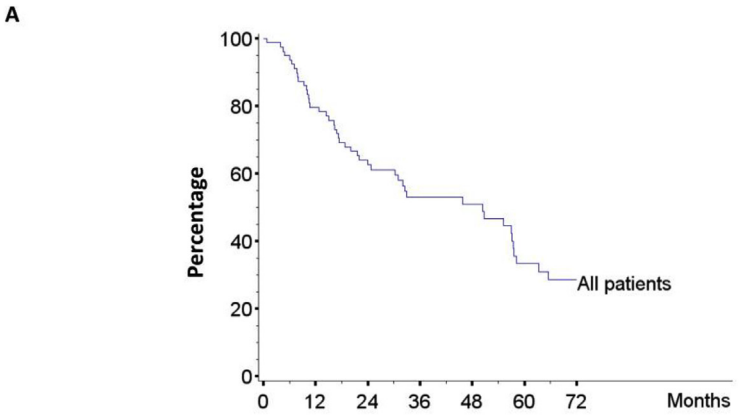
Setting M0 patients combined with Ki-67 < 3% as a reference, the HR for progression was 3.21 (95%CI 1.18–8.74) for M0 patients with intermediate Ki-67 values (3–20%). The HR raised to 5.06 (95% CI 2.29–11.2) for M1 patients with Ki-67 3–20% and 24.3 (95%CI 6.64–89.2) for M1 patients with Ki-67 > 20%. In M0 patients, median PFS was 145.7 months and 57.1 months for patients with Ki-67 < 3% and 3–20%, respectively (P < 0.0001). Overall, M1 patients with Ki-67 > 20% showed the worst prognosis (median PFS: 7.7 months) (Fig. 2).

Patient outcomes significantly differed according to tumor characteristics, along the lines summarized at the bottom of Fig. 1. In particular, M1 patients had a significantly lower PFS as compared to M0 patients (p = 0.0008), and the outcome was further stratified according to Ki-67 levels. While M0 patients with Ki-67 < 3% had excellent 3- and 5-year PFS of 83% and 64%, patients free of metastases with Ki-67 3–20% showed 3- and 5-year PFS of 53 and 27%, respectively. On the other hand, M1 patients with Ki-67 ≤ 20% had a 3- and 5-year PFS of 35% and 18%, respectively. None of the 4 patients with both synchronous liver metastases and Ki-67 > 20% was free of disease at the 3-year interval. When taking into account Ki-67 levels, irrespective of the presence of synchronous liver metastases, patients with Ki-67 < 3% had significantly better PFS than the ones with Ki-67 > 20% (p = 0.01). Notably, there were no significant differences in PFS according to tumor size ≤2 cm or >2 cm (p = 0.66).

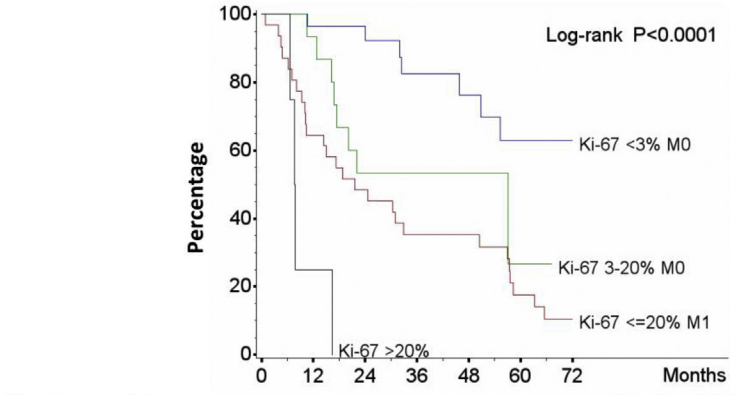
In the subgroup of M0 pNENs patients (N = 45), Ki-67 ≥ 3% was the best predictor of future liver metastases (HR 3.51; 95%CI 1.25–9.87) (data not shown) and the only factor associated with

Table 2
Univariable and multivariable analysis of risk factors associated with cancer progression.

	Univariable analysis		Multivariable analysis		Reduced model	
	N = 80		N = 76			
	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
Body/tail vs. head	1.18 (0.64–2.18)	0.60	1.09 (0.54–2.23)	0.81		
>2 cm vs. ≤2 cm	1.58 (0.88–2.84)	0.12	1.18 (0.58–2.39)	0.66		
Multifocal vs. unifocal	0.53 (0.13–2.19)	0.38	0.80 (0.19–3.49)	0.77		
Ki-67 ≥ 3% vs. <3%	**2.57 (1.43–4.61)**	**0.002**	**2.97 (1.26–7.02)**	**0.01**	**2.09 (1.16–3.76)**	**0.01**
MI ≥ 2 vs. <2	1.33 (0.75–2.35)	0.33	0.66 (0.27–1.56)	0.34		
Parenchymal invasion	**2.23 (1.11–4.49)**	**0.02**	1.43 (0.58–3.56)	0.44		
Vascular invasion	1.53 (0.86–2.75)	0.15	0.62 (0.28–1.37)	0.24		
Perineural invasion	**1.98 (1.11–3.53)**	**0.02**	1.32 (0.60–2.92)	0.49		
Necrosis of primary tumor	1.52 (0.71–3.26)	0.28	0.53 (0.21–1.35)	0.18		
Lymph node metastases	**1.90 (1.07–3.36)**	**0.03**	0.94 (0.45–1.97)	0.86		
Liver metastases	**3.63 (1.98–6.64)**	**<.0001**	**3.60 (1.70–7.61)**	**0.0008**	**3.20 (1.74–5.91)**	**0.0002**
Stage II vs. I (ENETS/AJCC)	1.96 (0.46–8.26)	0.36	–			
Stage III vs. I (ENETS/AJCC)	**4.18 (1.60–10.9)**	**0.003**	-			
Stage IV vs. I-III (ENETS/AJCC)	**5.24 (1.88–14.6)**	**0.001**	-			



Number at risk								Median PFS
All patients	80	62	45	30	24	15	9	50.5 months



Number at risk								Median PFS	HR (95% CI)
Ki-67 <3% M0	30	27	22	16	12	9	6	145.7 months	1.00
Ki-67 3-20% M0	15	14	8	4	2	1	0	57.1 months	3.21 (1.18-8.74)
Ki-67 ≤20% M1	31	20	15	10	10	5	3	21.6 months	5.06 (2.29-11.2)
Ki-67 >20%	4	1	0	0	0	0	0	7.7 months	24.3 (6.64-89.2)

Fig. 2. Progression-free survival (PFS) in 80 patients with surgically resected pNEN (Panel A) and according to combinatory classes made on Ki-67 status and presence of liver metastases (Panel B).

patients' survival (HR = 3.09; 95% CI 1.21–7.84) (Supplementary Table 1).

A high level of concordance in Ki-67 assessment between paired pre-surgical biopsies and surgical specimens (kappa 0.98; 95%CI: 0.94–1.00; Intra-class correlation: 0.99; 95%CI 0.98–0.99) was ascertained (Figs. 3 and 4) as well as between pathologists and automated counting (Supplementary Table S2). The Ki-67 group (<3%, 3–20%, >20%) was properly classified on pre-operative biopsy in 97.1% of cases (34/35), with misclassification occurring in only one (2.9%) patient only (3.0% in the biopsy *vs.* 2.6% in the surgical specimen).

Discussion

Surgical resection is the mainstay of treatment for patients with localized pNENs [15–17]. In clinical practice, the main preoperative criterion used to establish the malignant potential of pNENs and guide surgical decision is tumor size.

Neuroendocrine tumors ≤2 cm frequently represent an incidental finding and show a benign clinical course in up to 81% of cases [18]. However, recent reports suggest that the preoperative estimation of tumor size alone may not be a reliable predictor of malignancy [19,20]. According to a recent study, 36.6% of ≤2 cm pNENs surgically removed were histologically classified as G2/G3, and showed lymph node spread or distant metastases in 31% and 8% of cases respectively, resulting in survival rates similar to >2 cm pNEN patients [19]. Along this line, a recent systematic review suggested that, even if the malignancy rate in ≤2 cm pNENs was lower than in larger ones, a watch-and-wait policy did not provide any advantage because the risk of leaving a malignancy due to a conservative strategy was threefold higher than the benefits [20]. Taken together, these data indicate that pNENs may exhibit an aggressive behavior irrespective to their size and underline that there is an unmet need for reliable prognostic biomarkers in tumors ≤2 cm.

In the present study, we provided evidence that Ki-67 is a powerful prognostic marker irrespective of the primary tumor size. Based on Ki-67 preoperative index and presence/absence of liver metastases, 4 different prognostic classes of non-functioning pNENs were identified. It is worth noting that each class was associated with an incremental risk of tumor progression after tumor resection (Table 1). Similarly to studies on pharmacologic treatments of NENs, progression-free survival and risk of progression are the surrogate outcomes to assess a treatment benefit in

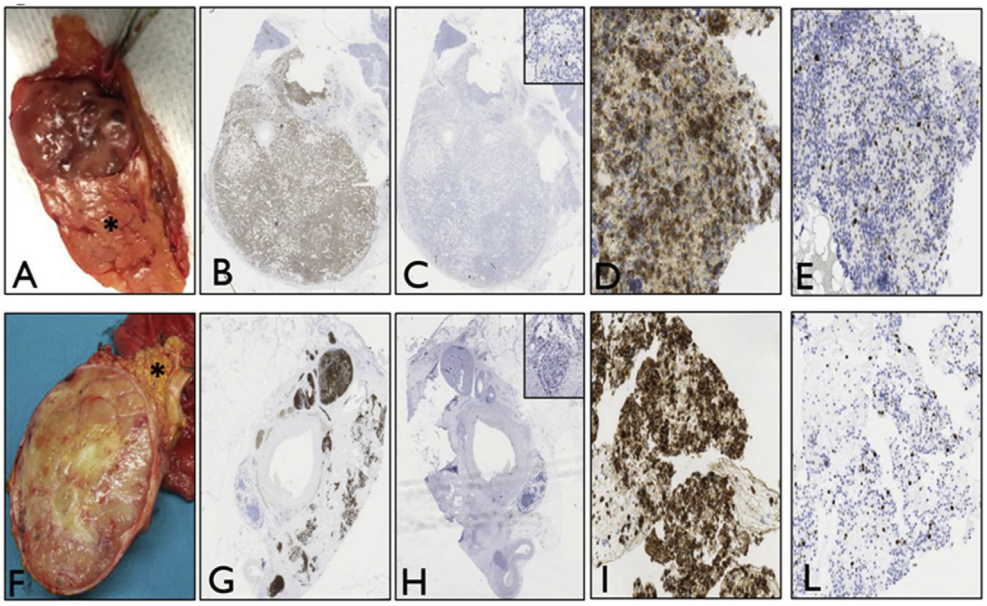


Fig. 3. Immuno-staining of surgical specimens and corresponding pre-surgical biopsies of pNENs ≤2 cm (1.6 cm: panel A–E) and >2 cm in size (4.2 cm: panel F–H). Panels A-C (A) Surgical Specimen, pNEN of 1.6 cm in size: the neoplasm is circumscribed by the normal pancreatic parenchyma (star); (B) diffuse staining for Chromogranin-A; (C) Ki-67 count is 1.2% in hot spots (C insert) according to WHO 2010; Panels D-E Pre-operative Biopsy of the pNEN shown in panels A–C; (D) diffuse staining for Chromogranin-A; (E) Ki-67 count is 1.1%; Panels F-H (F) Surgical Specimen, pNEN of 4.2 cm in size: notably, the normal pancreatic parenchyma is extensively occupied by the neoplasm, resulting in a thin residual area (star); (G) diffuse staining for Chromogranin-A; (H) Ki67 count is 1.4% in hot spots (H insert) according to WHO 2010; Panels I-L Pre-operative Biopsy of the pNEN shown in panels F–H; (I) diffuse staining for Chromogranin-A; (L) Ki-67 count is 1.3%.

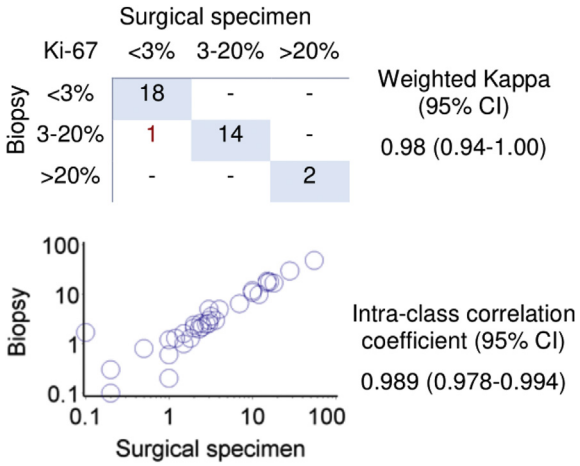


Fig. 4. Concordance (weighted Kappa) and intra-class correlation among different pathologists on pre-surgical pNEN biopsy and post-surgical pathology of the same tumor.

patients with NENs [21].

The class at most favorable prognosis consisted of patients with Ki-67 < 3% and no metastases: in this group none progressed in the 12 months after tumor removal, with a median time to progression of more than 12 years (145.7 months). On the other hand, the population of pNENs with Ki-67 > 20% (all presenting with liver metastases) had the worst prognosis and was likely to progress at a median of 7.7 months. In G1-G2 tumors with Ki-67 ≤ 20% and metastatic liver involvement, neither Ki-67 or tumor size were predictive of outcome. It should be noted, however, that Ki-67 was the best predictor of progression in patients with M0 disease and was the only significant predictor of OS.

Due to the clinical nature of the study, aiming at providing practical and easily reproducible prognostic indications, Ki67 was used as a categorical variable, according to the WHO guidelines. Despite some evidence supports the use of Ki67 expression as a continuous variable, further studies with larger sample size are needed in order to overcome the traditional categorization based on cut-offs in favor of a more comprehensive continuous variability of this biological marker [22].

Any intention-to-treat analysis on results of surgery in ≤2 cm primary tumor was beyond the scope of our study, because only patients considered at good risk-benefit ratio were selected for pNEN resection. With such limitation, our series however suggests that a sole biological surrogate (Ki-67) could be able to stratify pNENs prognosis, thus complementing the pure morphologic assessment. In light of the prognostic role of Ki-67, with high concordance of Ki-67 determination on pre-surgical biopsies with respect to surgical specimens, we suggest that pre-surgical evaluation of Ki-67 should be included in the algorithm guiding clinical decision on resection of pNEN of 1–2 cm in size. As risk of progression could be based on pre-operative Ki-67, observation and delayed surgery could be envisioned for patients with low Ki-67 (<3%) and no evidence of distant metastases. Similarly to other settings like lung and breast cancer, the systematic biopsy of primary pNENs, would aid in assessing the biological characteristics for proper clinical interventions.

EUS-FNA has been established as the technique of choice for sampling solid pancreatic masses with a sensitivity, specificity, positive and negative predictive value of 85%, 99%, 99% and 65%, respectively [23–25]. Our findings confirm such data, with a good correlation between Ki-67 values derived from EUS-FNA pre-operative biopsy and surgical specimens of 1–2 cm pNENs. This supports the pivotal role of EUS-FNA in the diagnosis and grading of pancreatic NENs. Our result was favored by the fact that none of our resected ≤2 cm pNEN measured less than 1 cm. Very small pancreatic lesions <1 cm are not invasively studied in our Center, unless other signs of malignancy are suspected (i.e. positive lymph-nodes at nuclear imaging studies, presence of carcinoid syndrome) [5,6,18,21,26–28].

Our study has several limitations. Firstly, it is a retrospective series enrolling only patients who underwent surgical resection. This impeded a thorough intention-to-treat analysis and any definite conclusion on those patients judged unsuitable to interventional/surgical therapies. With this respect, no information could be collected about non-resected, more advanced patients and on the possible differential influence of Ki-67 and tumor burden on response to non-surgical treatments. Secondly, despite a significant difference in risk of progression across the 4 classes indicated in Fig. 2, we were unable to show corresponding differences in overall patients' survival. This may be due to the relatively short median follow-up but was likely related to differentiated eligibility of the patients to curative treatments for liver metastases such as liver resection, ablation and liver transplantation. All these treatments have been shown to significantly prolong survival.

Conclusions

Ki-67 and presence of liver metastases are the major prognostic factor in pNENs and identify classes at different progression risks. These two factors were predictors of prognosis independently from tumor size. Although stemming from a retrospective series and therefore requiring validation in prospective studies, our data suggest that tumor proliferation Ki-67 index is a powerful prognostic tool to be assessed in the preoperative setting of pNEN.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ejso.2018.10.052.

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