Pancreatic Neuroendocrine Tumors: Accurate Grading With Ki-67 Index on Fine-Needle Aspiration Specimens Using the WHO 2010/ENETS Criteria

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BACKGROUND: The natural history of pancreatic neuroendocrine tumors (panNETs) is extremely variable. One of the most controversial problems in diagnosis is the accurate prediction of the clinical behavior of these tumors. PanNETs that behave aggressively with a malignant course may have bland cytologic features, while some tumors with previously described "malignant" features may behave in a benign or indolent fashion. Various classification schemes have been proposed for grading panNETs. The European Neuroendocrine Tumor Society (ENETS) and 2010 World Health Organization (WHO) classification schemes include counting the mitotic index and/or the Ki-67 proliferation index for grading. The current study was undertaken to determine whether tumors sampled by endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) can be accurately graded based on the Ki-67 index when compared to surgical samples. **METHODS:** Corresponding EUS-FNA cytology and surgical tissue specimens were obtained for 22 tumors and stained for hematoxylin and eosin (H&E) and the Ki-67 proliferation marker (MIB-1 antibody). Samples were graded by scoring Ki-67 staining indices in accordance with the 2010 ENETS/WHO criteria. The grading scores assigned to the EUS-FNA cytology samples were compared with the scores assigned to the corresponding histological samples. **RESULTS:** The majority (86%) of EUS-FNA cytology samples and corresponding surgical tissue specimens demonstrated concordant grading based on Ki-67 indices. **CONCLUSIONS:** These results indicate that EUS-FNA cytology samples can be accurately graded based on the WHO Ki-67 labeling scheme. Thus, Ki-67 scoring in EUS-FNA cytology samples is an alternative approach for establishing the grade of panNETs. Accurate grading of panNETs is critical for predicting tumor biology, patient prognosis, and making informed decisions regarding patient management and treatment. ***Cancer (Cancer Cytopathol)*** **2014;122:770-8.**

KEY WORDS: pancreatic neuroendocrine tumor; fine-needle aspiration; Ki-67; cytology; grading; proliferation index.

INTRODUCTION

Pancreatic neuroendocrine tumors (panNETs) are relatively uncommon (accounting for <3% of all pancreatic neoplasms) and typically occur in adults without a significant gender predilection.[1-4] Most tumors occur as solitary, sporadic lesions and a minority are associated with inherited familial syndromes.[1,2] PanNETs demonstrate cytological and morphological heterogeneity and significant variability with respect to clinical outcome. They are often identified because of symptoms related to the increased secretion of endocrine hormones from tumor cells. However, up to 40% of panNETs are nonfunctioning tumors and do not secrete measurable levels of hormones, making them difficult to detect and leading to their identification at late stages of disease secondary to obstruction or abdominal discomfort related to the growing mass.[5,6]

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The clinical behavior of panNETs has been described as highly unpredictable, since the morphologic features do not necessarily reflect clinical outcomes.[7,8] The strongest predictors of survival are disease stage and tumor grade, making early diagnosis extremely important.[1,9] In general, morphologic examination is performed to distinguish well-differentiated, low-grade tumors from poorly differentiated, high-grade tumors.[10] Multiple classification schemes have been proposed for grading panNETs.[2,8] The classification system currently recommended by the World Health Organization (WHO) is based on the guidelines issued by the European Neuroendocrine Tumor Society (ENETS). The WHO classifies panNETs according to tumor histopathology, proliferative activity, site of origin, invasion, and metastases. Proliferation is determined by measuring the mitotic activity and Ki-67 index (Table 1).[1,9,11] Many studies suggest that the Ki-67 index is related to the malignant potential and clinical behavior of panNETs and may be an important prognostic indicator.[4,7,8,12]

The morphologic features and proliferative indices of panNETs have been previously evaluated in cytology studies.[3,12] However, these studies were performed before the current WHO guidelines and one of the studies[12] was performed on cytology smear slides rather than cell block specimens. The current study was undertaken to determine whether grading of tumors based on the current WHO/ENETS recommendations can be accurately performed on cytological cell block samples obtained by endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA).

MATERIALS AND METHODS

Institutional Review Board approval was obtained for the current study. A retrospective review of the institutional pathology archives over a 13-year period was performed to identify primary panNETs. A total of 32 cases with EUS-FNA cytological material were identified. Of those cases, 25 had correlating surgical specimens available for evaluation. Formalin-fixed paraffin-embedded cell blocks from these 25 cases were evaluated to assess for adequacy of material, arbitrarily set at >100 tumor cells in the cell block sample. Based on this definition, adequate cell block samples were available for 22 cases. Representative tissue blocks of the corresponding surgical specimens were also obtained. Tissue blocks were selected based on review of the hematoxylin and eosin (H&E)-stained sections for the highest mitotic index in comparison with other sections from the same surgical specimen. Neuroendocrine differentiation was verified by immunohistochemical staining for the neuroendocrine markers antichromogranin A (LK2H10, mouse monoclonal; Cell Marque, Rocklin, Calif) and/or antisynaptophysin (Z66, rabbit polyclonal; Invitrogen, Carlsbad, Calif) using standard techniques. Immunohistochemical staining for antitrypsin (mouse monoclonal; Chemicon International, Temecula, Calif) was also used in one case to exclude acinar differentiation.

Two unstained slides from each cytological cell block and each histological surgical tissue block were obtained for immunohistochemical staining. One slide from the cytology sample and 1 slide from the surgical histology sample from each case were stained with H&E to evaluate morphology. The remaining cytology and histology slides for each case were stained with MIB-1 antibody (1:1000 dilution; Dako, Carpinteria, Calif) to assess Ki-67 proliferation. The mitotic index was calculated on the H&E sections for the histology samples according to the 2010 WHO guidelines. Ki-67 indices for the cell block sections were calculated as the total number of tumor cells with positive nuclear staining divided by the total number of tumor cells present. Ki-67 indices for histological sections were calculated as the percentage of tumor cells with positive nuclear staining in 2000 cells counted in the highest area of nuclear labeling as indicated by the WHO/ENETS guidelines.[1] For cases in which there was discordance between the mitotic and Ki-67 grade on histology samples, the higher grade was assigned according to the WHO/ENETS recommendations.[1]

Two pathologists performed all cytological and histological evaluations in a blinded fashion. Cases in which there were discrepancies between the two pathologists were evaluated by a third pathologist. Agreement between cytological grade and histological grade was evaluated using percent agreement and *k*-statistics.[13]

RESULTS

Clinical Characteristics

Clinical characteristics are summarized in Table 2. The patient population included 9 men and 13 women. The age at diagnosis ranged from 23 years to 77 years, with a mean age of 54 years. The localization of tumors varied, with 41% of tumors located in the pancreatic head, 36% in the pancreatic tail, and 23% in the pancreatic body.

Table 1. WHO Neuroendocrine Tumor Classification

WHO 2010			WHO 2004	
Grade	Mitoses Per 10 HPF[a]	Ki-67 Index, %[b]	Grade	Definition
Neuroendocrine tumor, grade 1	<2	≤2	Well-differentiated endocrine tumor. 1.1: Benign behavior.	Confined to the pancreas, <2 cm in diameter, ≤2 mitoses per 10 HPF, ≤2% Ki-67-positive cells, no angioinvasion or perineural invasion.
			Well-differentiated endocrine tumor. 1.2: Uncertain behavior.	Confined to the pancreas and ≥1 of the following features: 2 cm in diameter, >2 mitoses per 10 HPF, >2% Ki-67-positive cells, angioinvasion, perineural invasion.
Neuroendocrine tumor, grade 2	2-20	3-20	Well-differentiated endocrine carcinoma. Low-grade, malignant.	Macroscopic local invasion and/or metastases.
Neuroendocrine tumor, grade 3 (NEC)	>20	>20	Poorly differentiated endocrine carcinoma. High-grade, malignant.	>10 mitoses per 1 HPF.

Abbreviations: HPF, high-power field; NEC, neuroendocrine carcinoma; WHO, World Health Organization.
[a] HPF is 2 mm^2; at least 40 fields are evaluated at ×40 magnification in the area of highest mitotic density.
[b] Percentage of 2000 tumor cells in the area of highest Ki-67 nuclear labeling.

TABLE 2. Clinical Data and Proliferation Indices

Clinical Characteristics							Procedure Assessment		Ki-67 Index			
									Cytology		Histology	
Case No.	Sex/Age, Years	Location	Size, cm	Functional Status	Treatment Modality	Follow-Up, Months/ Status[a]	CP Present	No. of Passes	Ki-67, %	Grade	Ki-67, %	Grade
1	Woman/41	Head	2.4	NF	R	7/ANR	Yes	4	<1	1	<1	1
2	Man/55	Body	4.0	FC	R	NA	Yes	5	<1	1	<1	1
3	Man/77	Tail	1.5	NF	R	31/ANR	Yes	5	<1	1	<1	1
4	Woman/43	Head	4.6	NF	R	169/ANR	Yes	4	<1	1	<1	1
5	Woman/62	Body	3.2	NF	R	45/ANR	Yes	5	<1	1	<1	1
6	Man/60	Body	1.5	NF	R	3/ANR	Yes	8	<1	1	<1	1
7	Woman/63	Body	2.2	FC	R	28/ANR	Yes	1	1	1	<1	1
8	Woman/41	Head	1.8	NF	R	16/ANR	Yes	8	1	1	1	1
9	Man/41	Body	3.3	NF	R	3/ANR	Yes	7	1	1	1	1
10	Woman/69	Tail	1.8	NF	R	28/ANR	Yes	5	<1	1	2	1
11	Man/41	Tail	1.6	NF	R	NA	Yes	5	2	1	<1	1
12	Man/63	Head	1.8	FC	R	68/ANR	Yes	3	2	1	2	1
13	Man/61	Body	1.5	NF	R/Chemo	20/ANR	Yes	9	1	1	3	2
14	Woman/62	Tail	1.6	NF	R	32/ANR	Yes	2	4	2	1	1
15	Woman/56	Head	1.8	FC	R	7/ANR	Yes	3	4	2	4	2
16	Man/23	Head	2.6	NF	R	22/ANR	Yes	2	3	2	4	2
17	Woman/64	Head	3.0	NF	R/Chemo	8/ANR	Yes	3	3	2	5	2
18	Woman/53	Body	4.5	FC	R/Chemo	12/LM	Yes	9	4	2	3	2
19	Woman/62	Tail	8.2	NF	R/Chemo	11/ANR	Yes	4	8	2	7	2
20	Woman/48	Head	3.0	NF	R	8/ANR	Yes	7	13	2	13	2
21	Woman/50	Tail	6.0	NF	R/Chemo	25/LM and DE	Yes	5	3	2	26	3
22	Man/48	Tail	4.8	NA	R/Chemo	52/LM	Yes	8	36	3	27	3

Abbreviations: ANR, alive with no residual disease; Chemo, chemotherapy; CP, cytopathologist; DE, deceased; FC, functioning; LM, liver metastasis; NA, not available; NF, nonfunctioning; R, resection.
[a] From the time of the initial diagnosis on cytology to death or date of last follow-up.

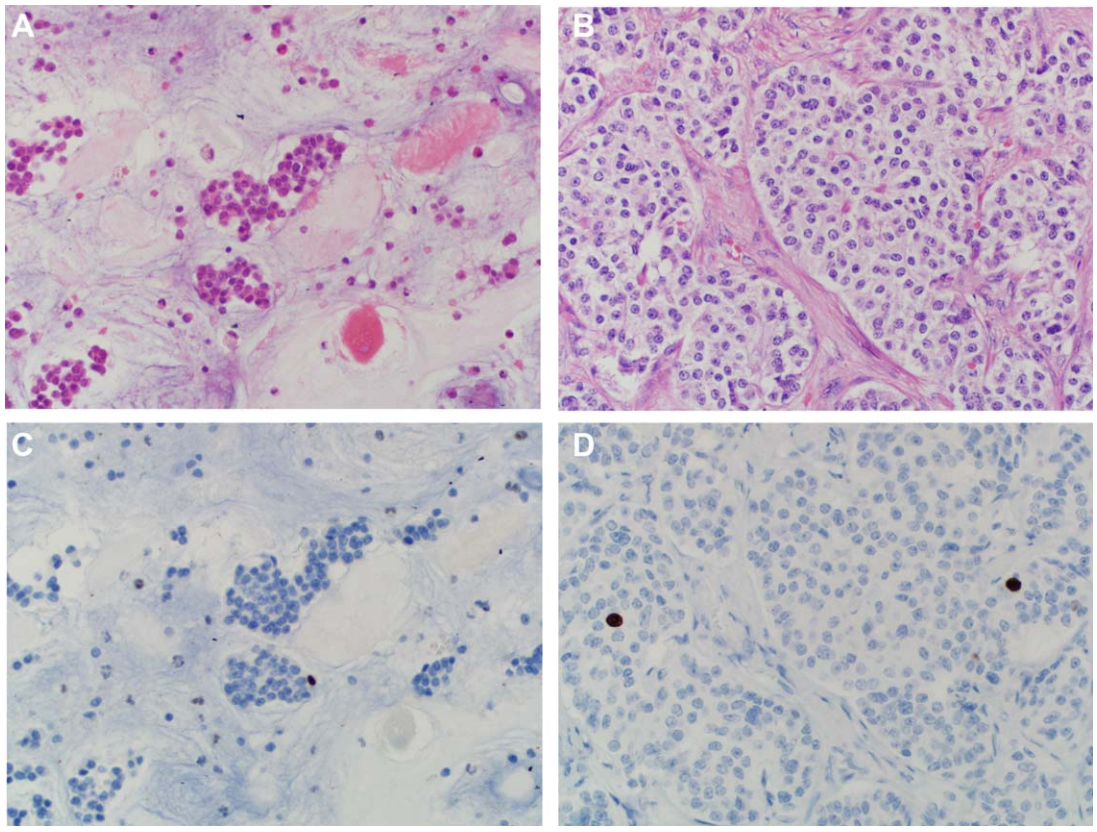

Figure 1. (A) A grade 1 pancreatic neuroendocrine tumor is shown in a cell block section derived from endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) material and (B) the corresponding surgical specimen (H & E stain, original magnification × 400). (C) A cell block section derived from EUS-FNA material and (D) the corresponding surgical specimen are shown (MIB-1, original magnification × 400).

Tumor functional status was available in 95% the cases and of those, 76% were nonfunctioning. The overall tumor size ranged from 1.5 cm to 8.2 cm, with an average size of 3 cm. Needle size for EUS-FNA sampling ranged from 19-gauge to 25-gauge; the number of EUS-FNA passes ranged from 1 to 9 passes. Rapid on-site evaluation by a cytopathologist was performed in all cases. Treatment consisted primarily of surgical resection. Approximately 27% of patients were treated with adjuvant chemotherapy before surgery, the effects of which, in regard to tumor grading, have to our knowledge not been studied to date. Patient follow-up ranged from 7 months to 169 months and the majority of patients were alive without residual disease at the time of last follow-up. Three patients developed liver metastases, 2 patients were lost to follow-up, and 1 patient died of disease.

Mitotic Index

Mitotic grade was found to be discordant with the Ki-67 grade on histological samples in 3 of 22 cases. In all cases, the mitotic grade was lower than the Ki-67 grade, and therefore the higher grade was assigned to the tumors (Table 2).

Ki-67 Index

The percent agreement of grading scores based on Ki-67 proliferation rates between cytological and histological samples was 86%. The k statistic was 0.74 (95% confidence interval, 49.9-100; $P = .00003$), suggesting good agreement (Table 2).[14] Twelve of 22 cases (55%) with Ki-67 indices $\leq$2% were interpreted as grade 1 (Fig. 1). Six of 22 cases (27%) were scored as grade 2 based on Ki-67 indices ranging from 3% to 20% (Fig. 2). One case was designated as a grade 3 tumor due to a Ki-67 index >20% (Fig. 3). Because grade 3 panNETs are rare, an additional stain for trypsin was performed on the cell block and histological sections of this case to exclude acinar cell differentiation and was found to be negative. Three cases demonstrated discordance between the cytologic and histologic grades. Case 13 was interpreted as grade 1 based

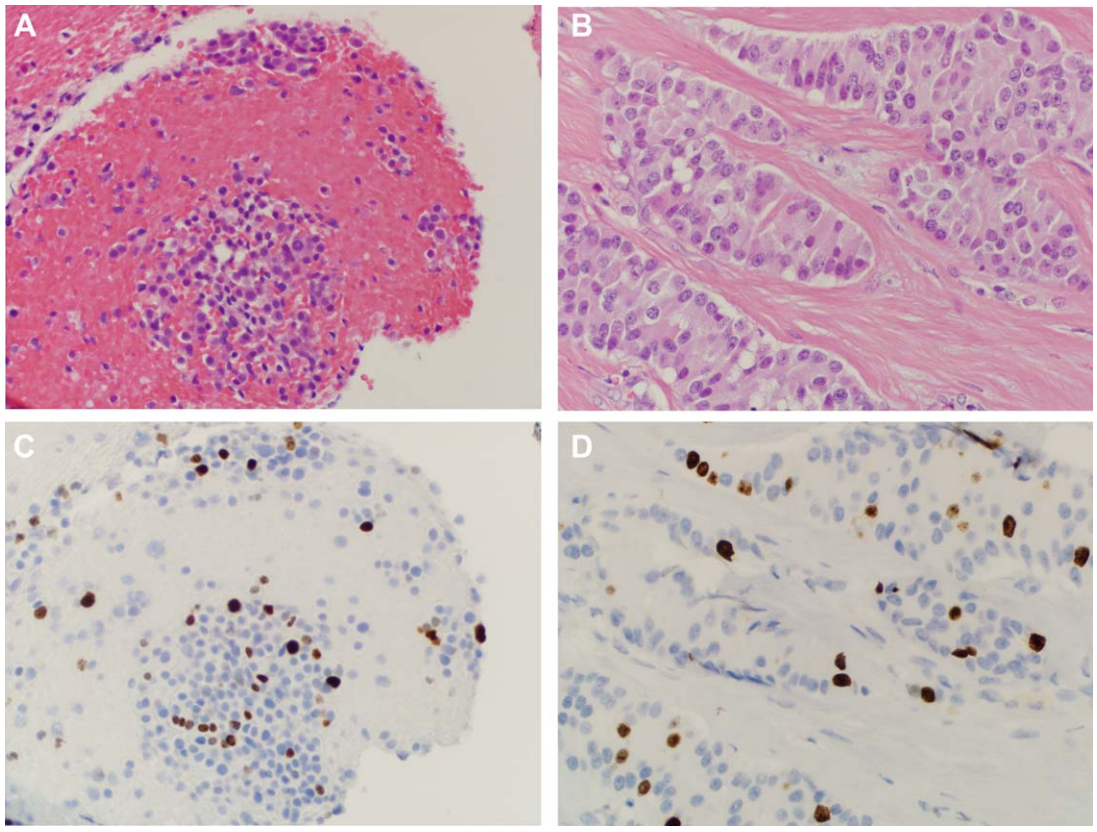

Figure 2. (A) A grade 2 pancreatic neuroendocrine tumor is shown in a cell block section derived from endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) material and (B) the corresponding surgical specimen (H & E stain, original magnification × 400). (C) A cell block section derived from EUS-FNA material and (D) the corresponding surgical specimen are shown (MIB-1, original magnification × 400).

on cell block material but was scored as grade 2 by histological evaluation. Case 14 was interpreted as grade 2 based on cell block material and grade 1 by histological evaluation. Finally, case 21 was scored as grade 2 on cytological examination and grade 3 by histological evaluation.

DISCUSSION

The results of the current study provide evidence that WHO grading of panNETs can be performed on cytologic samples by assessing the Ki-67 index on cell block material and that there is good agreement between the 2010 WHO/ENETS grade obtained through the evaluation of Ki-67 index on EUS-FNA cytology material when compared with that obtained on surgical resection specimens.

Several factors have been proposed to be predictors of malignant potential for panNETs. Regional lymph node involvement, tumor size >2 cm, and tumor proliferation rates are among the most important factors found to be predictive of aggressive tumor behavior.[10] A growing body of evidence suggesting that the proliferation rate is linked with tumor biological behavior[15-17] has led to the establishment of grading guidelines by the 2010 WHO and ENETS for panNETs based on mitotic count and the Ki-67 proliferative index. However, to our knowledge, the best method to determine tumor grade (ie, mitotic index vs Ki-67 index) in panNETs remains controversial, and the recommendations set forth by the WHO/ENETS schema for the histological evaluation of tumor grade have not been tested on cytological specimens.

According to the WHO guidelines, the recommended method for determining the mitotic count is to count 50 high-power fields or a 2 mm^2 area and obtain an average count per 10 high-power fields. This is challenging in cytological material not only due to the limitations in the availability of tumor tissue but also because aspiration

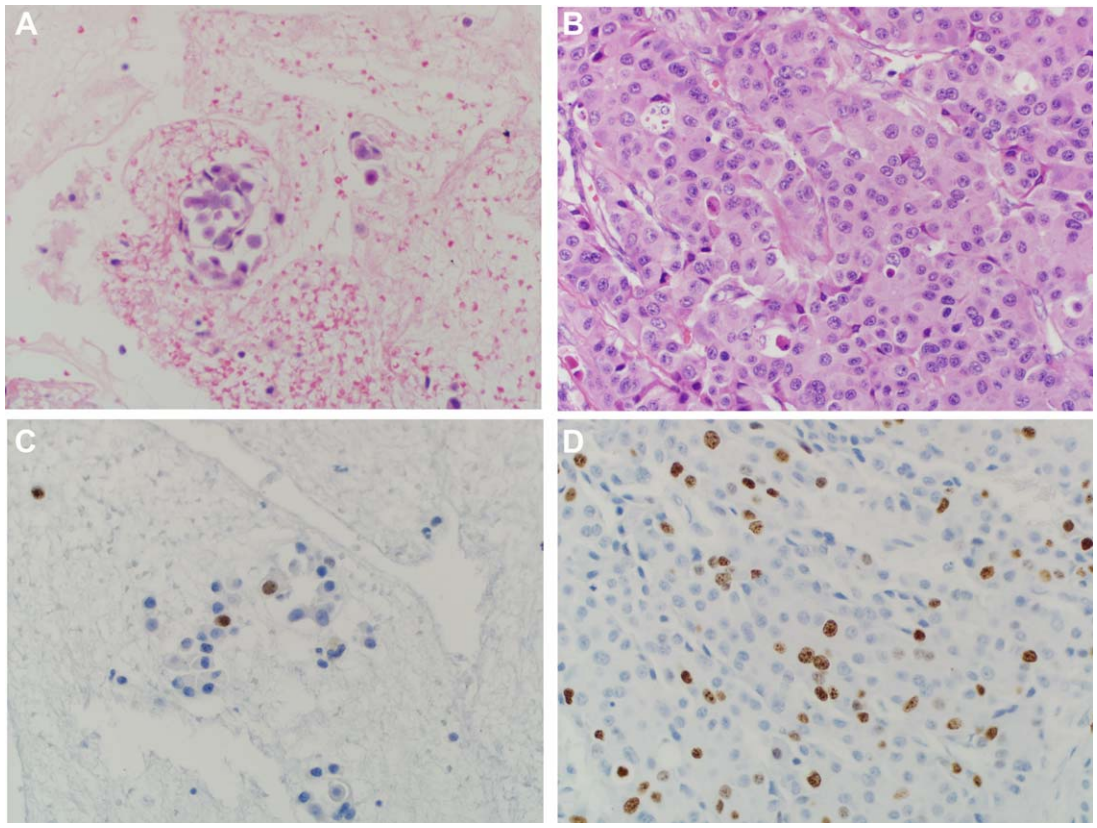

Figure 3. (A) A grade 3 pancreatic neuroendocrine tumor is shown in a cell block section derived from endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) material and (B) the corresponding surgical specimen (H & E stain, original magnification × 400). (C) A cell block section derived from EUS-FNA material and (D) the corresponding surgical specimen are shown (MIB-1, original magnification × 400).

samples typically represent various regions sampled within a single tumor. For these reasons, the mitotic index as a measure of the proliferation rate in small tissue samples is imprecise and was therefore excluded as a grading method on the cytological samples in the current study. Grading according to the mitotic and Ki-67 indices can sometimes be discordant. In such instances, the recommendation is to assign the higher grade.[1] Studies have shown that tumors are more often undergraded using the mitotic index compared with the Ki-67 index, as was observed in 14% of the histology cases in the current study, and that the Ki-67 index correlates significantly and independently with the clinical outcome of patients with panNETs.[8,15,18,19] One explanation for undergrading by mitotic index may be that counting mitotic figures is a subjective process and immunostaining allows for the easier and more efficient recognition and identification of proliferating tumor cells. McCall et al[18] suggested that because the mitotic rate represents the proliferation per unit area, characteristics such as tumor cell size or the amount of stroma within a given section may influence its assessment; because Ki-67 is present in the G1 (Gap 1), G2 (Gap 2), and the S (Synthesis) phase of the cell cycle in addition to the M (Mitosis) phase, immunostaining may demonstrate positivity for Ki-67 when mitoses are absent.[18,19] In their study, greater than one-third of panNETs designated as grade 1 according to the mitotic index were upgraded to grade 2 by the Ki-67 index, whereas only a small minority of tumors designated as grade 1 according to the Ki-67 index were mitotic grade 2. The overall survival in the former group was similar to that of patients with mitotic grade 2 tumors, whereas the latter group failed to demonstrate any significant clinical or histopathological difference from those patients with mitotic grade 1 tumors.[18]

The WHO/ENETS recommendations for the calculation of the Ki-67 index is to manually count Ki-67-positive tumor cells in 2000 tumor cells in the area of highest nuclear labeling or "hot spot" in tissue sections. In

the cell block samples in the current study, we calculated this index by dividing the number of tumor cells with positive nuclear staining by the total number of tumor cells present. Some studies on histological material have considered an "eyeballed" estimate to be adequate.[18] However, a recent study by Tang et al[20] concluded that an "eyeballed" estimate is not appropriate for assessing the proliferation index of gastrointestinal and pancreatobiliary tract NETs and that digital image analysis and manual counting are the appropriate methods with which to assess Ki-67 labeling.[20] The loss of architecture and inherent sampling limitations in cytological material pose challenges in determining the tumor "hot spot" in cytological material. Variations in practice settings and expertise among different operators in evaluating Ki-67 rates and distinguishing between Ki-67-positive tumor cells and inflammatory cells present further limitations. Less subjective assessment may be possible with computer-aided image analysis, such as that proposed by Remes et al,[21] in which surgically resected histological samples of pancreatic and ileal NETs were assigned Ki-67-based tumor grades with the assistance of an image analysis software program.[21]

In the current study, three cases demonstrated discordance between the cytological and histological grades. Two of these were undergraded and one case was overgraded based on cytology. Due to tumor heterogeneity in panNETs, Ki-67 immunoreactivity can be focal, and EUS-FNA sampling may not be representative of the most proliferative area within a tumor, leading to undergrading of the tumor. Conversely, because various regions of a tumor may be sampled by EUS-FNA, it is possible to aspirate >1 proliferative "hot spot," leading to overgrading of the tumor. Another limitation associated with EUS-FNA sampling is procedural operator dependence. Less experienced operators may have difficulty obtaining a representative cellular sample that can be processed as a cell block for Ki-67 immunostaining, particularly in the setting of small tumors or those that have undergone cystic degeneration.

Results of a study by Larghi et al,[22] in which EUS-FNA cytological samples of nonfunctioning panNETs stained with MIB-1 to assess the Ki-67 proliferation rate were used to establish tumor grade, directly support the results of the current study. In their study, 10 of 12 patients (83%) who underwent subsequent surgical resection demonstrated concordant preoperative and postoperative tumor grades based on the Ki-67 indices. However, the details of their analysis and the minimum number of cells evaluated to obtain a proliferation index on the EUS-guided samples were not specified. Piani et al showed an agreement rate of 78% between Ki-67 expression by immunocytochemistry and immunohistochemistry; however, the cytological evaluation in their study was performed on cytological smears instead of cell block material and details regarding smear cellularity and the number of cells counted were not provided.[12] In a study by Alexiev et al, cytological cell block material obtained from 15 patients was evaluated for the Ki-67 index using automated quantitative image analysis software; however, correlation with surgical material was not performed.[15]

Small study sample size, particularly for grade 2 and grade 3 tumors, is a limitation that plagues many studies involving panNETs. Several reasons may account for this, among them: 1) high-grade panNETs are rare[1,2,17]; 2) patients with high-grade panNETs are treated with chemotherapy or placed in drug trials without undergoing surgical resection of the tumors, or the tumors are unresectable due to patient comorbidities or advanced disease; 3) of the tumors resected, some are without prior FNA assessment or the FNA is performed at another facility before surgical management; and 4) cases for which FNA material is available may not always have adequate cell block material for additional evaluation by immunostaining.

Multiple medical therapies are available for treatment of patients with panNETs. Although localized panNETs are primarily treated with surgical resection, ablative and embolization techniques are typically used for the management of liver metastases.[23,24] Tumor location, grade, stage, and proliferative index play a role in selecting the appropriate treatment regimens.[25] Somatostatin analogues, chemotherapy, and targeted agents are the main pharmacological treatments available for patients with panNETs. Clinical trials evaluating somatostatin analogues in conjunction with interferon-α for patients with advanced disease and their application in grade 1 panNETs is currently ongoing.[24-26] Chemotherapy modalities can be used in patients with unresectable tumors or as an adjunct therapy for tumor debulking; however, toxicity limits their use.[25,26] Newer agents such as everolimus and sunitinib can be considered in patients with advanced grade 2 and grade 3 panNETs that are refractory to chemotherapy or as an alternative treatment

to ablative therapy or chemotherapy.[24-26] Patients with localized grade 1 panNETs have also demonstrated responses to these newer therapies but their usefulness in patients with low-grade disease is not clearly defined and such therapies are not recommended if complete surgical resection can be performed.[23,25] Overall, chemotherapy is not used in patients with resectable grade 1 and grade 2 tumors but can be considered in patients with grade 2 and grade 3 tumors demonstrating advanced disease.[25]

The results of the current study demonstrate that calculation of the Ki-67 proliferation index in cytological material shows good agreement with that obtained by histological material. Thus, stratification of patients according to tumor grade can be done by Ki-67 assessment in EUS-FNA samples, allowing for more informed decisions to be made regarding the clinical management of patients. This is particularly critical for those patients who have comorbidities that may limit treatment with certain types of interventions and may serve as a potentially valuable tool in the preoperative assessment of patients. Larger studies that include a greater number of patients with high-grade tumors should be performed to validate and standardize Ki-67 evaluation in EUS-FNA cytologic samples as an important strategy for establishing tumor grade and prognosis in patients with panNETs.

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CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

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