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Diagnostic Yield of EUS-FNA with 22G Needles for the Assessment of the Ki-67 Index in Pancreatic Neuroendocrine Tumors

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ABSTRACT

Background: the Ki-67 antigen is a marker whose expression is strictly associated with cell proliferation and only can be detected in the nucleus. Its index correlates with survival in patients with pancreatic neuro endocrine tumors. The aim of this study was to evaluate whether endoscopic ultrasound-guided fine needle aspiration with 22G needles, allows obtaining adequate samples for the assessment of the Ki-67 index and adapting the gradation of this type of tumors to the new classification of WHO-2010.

Methods: retrospective observational analysis of a database of 20 patients with pancreatic neuro endocrine tumors (mean age 56 years ± 15) with an average of lesion size of 24.5 mm (Interval: 10-60 mm), who underwent a fine-needle aspiration puncture guided by endoscopic ultrasonography with 22G needles, to obtain samples for cytology, immunocytochemistry and Ki-67 assessment.

Results: the cytology was positive in 100% of cases. Positive immuno cytochemistry had a diagnostic sensitivity of 90% (18/20). The Ki-67 index was assessed in 80% of cases (95% CI: 56%-94%) (16/20). The correlation with the surgical samples was of 80% (8/10). Only two cases had to be reevaluated. There were no significant differences between the Ki-67 value obtained pre and post-surgery (p <0.669).

Conclusions: the procedure showed a high sensitivity in the cytological confirmation and a high accuracy (80%) in the assessment of the Ki-67 index, with no adverse events attributable to the puncture.

Keywords: Endoscopic ultrasonography; Fine-needle aspiration puncture; Ki-67 antigen; Gastro-enteropancreatic neuroendocrine tumor; Immunocytochemistry; Tissue diagnosis; Pancreas

INTRODUCTION

Pancreatic neuroendocrine tumors (**PNETs**) are among the most common neuroendocrine tumors (**NET**) and most are indolent, but have a potential for malignancy. Are relatively rare tumors and comprise 1-2% of all pancreatic neoplasms. They are divided into functioning and nonfunctioning tumors, being the latter approximately 90% of all classified. There are familial and sporadic genetic causes for its appearance that are well known, but its molecular pathogenesis remains unknown and its individual biological behavior is unpredictable [1,2]. Having a high tumor grade, lymph node, liver metastasis, and large tumor size generally indicates a less favorable prognosis [3].

The PNETs new gradation of WHO of 2010, which classifies them into well differentiated tumors (grade 1, G1 and grade 2, G2) and poorly differentiated (grade 3, G3) tumors, is based on the ki-67 labeling index and Mitotic count. The first studies on the quantification of Ki-67 placed cutoff in 5% [4], although later was < 2%, [5] and finally 2-3% (2.8%) [6] (Table 1).

Table 1: Classification and grading of TNEP OMS-2010.

Classification / Degree	Mitotic index (per 10 hpf)	Ki67 index (in%)
TNE-G1	<2	<3
TNE-G2	20-Feb	20-Mar
CNE-G3	>20	>20

TNE: Neuroendocrine tumor; CNE: Neuroendocrine carcinoma; Hpf: High power field; 10hpf = 2 mm2, at least 40 fields (with an increase of 400 ×) evaluated in areas of higher mitotic density.

The Ki-67 antigen is a marker whose expression is strictly associated with cell proliferation and is only detected in the nucleus. Now is known that the index correlates with survival in patients with neuroendocrine tumors. Preoperative detection of Ki-67 expression may also help to determine both, the best timing of surgery and the extent of surgery in case of single pancreatic lesions. In patients with multiple life-threatening NEM-1, it may also help to optimize the timing and extent of the surgical technique.

Endoscopic ultrasonography (**EUS**) in addition to being one of the imaging techniques capable of diagnosing tumors ≤ 1 cm in diameter [7], allows guiding fine-needle aspiration (**FNA**) to obtain optimal material for cytology and immunocytochemistry (**ICQ**) and confirm the diagnosis of PNET, with an accuracy close to 90%; being also a technique that would meet the requirements of low risk of bleeding and low economic cost [8-10] (Figure 1).

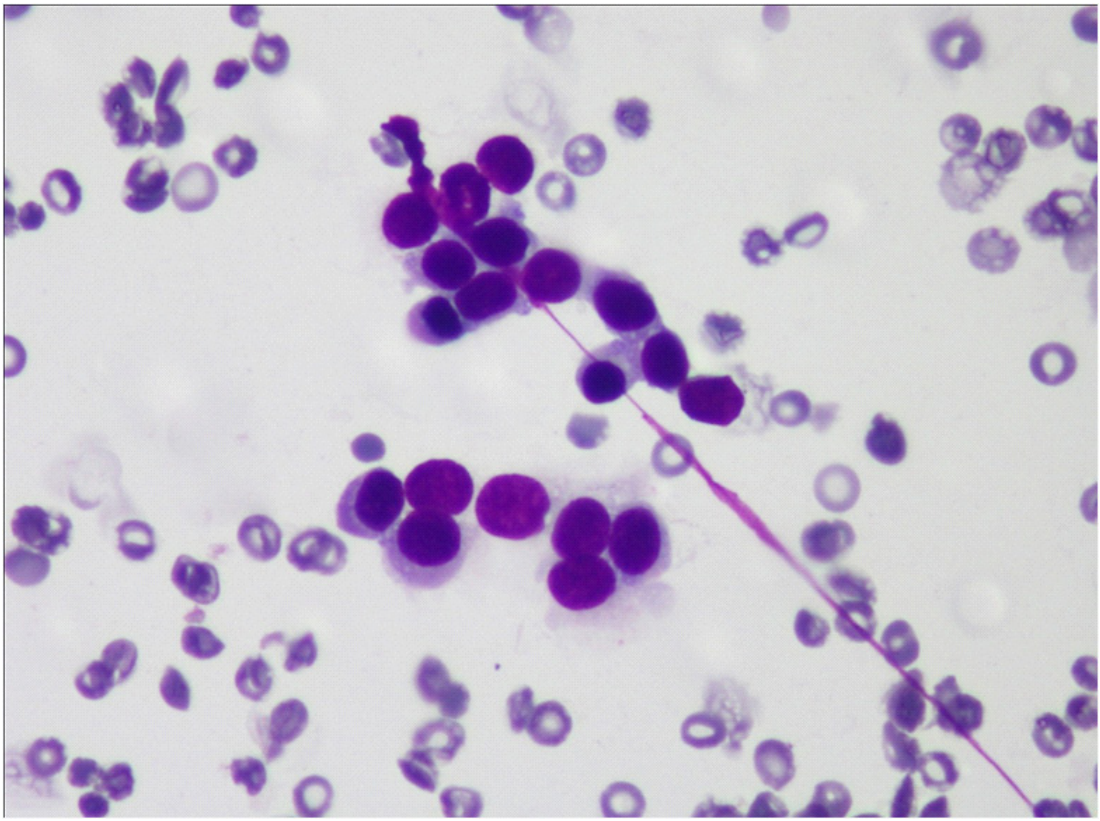

Figure 1: Cells forming small nests or chains with scarce cytoplasm and uniform rounded nuclei (DQ for 400). Cytology obtained by USE-PAAF 22 G of a TNEP.

The limitations of EUS would be the high dependence of the operator and the results are somewhat worse in case of tumors located in the pancreatic tail or in ectopic tumors [11].

The aim of the present study was the analysis of the accuracy of Ki-67 labeling index results in the PNET samples obtained using endoscopic ultrasound-guided fine-needle aspiration (**EUS-FNA**) with 22 G needles, to establish their classification according to WHO-2010 classification and to be able to reliably evaluate the prognosis of patients.

MATERIALS AND METHODS

For patients selection, a retrospective, observational analysis of a database of three centers with local Institutional Review Board (**IRB**) was carried out following the established protocols for access to data and management of clinical records. Of the 70 cases of PNET included in the database, 28 (40%), registered between January 2008 and June 2015, were selected. The mitotic index and the Ki-67 index were studied. Of these 28 cases, those who had undergone an EUS-FNA (22 G needles) of TNEP (Echotip 3-22 Ultra Flexible, Cook Medical, Bloomington, Indiana (USA)), according to conventional technique (aspiration with negative pressure of 5-10 mL), were studied (n = 20 cases). At 50% (n = 10 cases), there was surgically confirmed.

The inclusion criteria for the EUS-FNA were: patients with a diagnostic orientation of PNET by USE, doubtful or of non-functioning tumor. All EUS-FNA procedures were performed after signing the informed consent, with previous coagulation tests, and with sedation (propofol), performed by an anesthesiologist.

In 80% of the samples, microsatellite instability and Ki-67 mitotic index were assessed, in order to evaluate the benignity or malignancy of the NPT as well as the prognosis, with a correlation of 80% in the operated patients (8/10).

The EUS-FNA was performed with two linear devices: Fuji 530 UT. Tokyo (Japan) and Pentax FG-38UX. Tokyo (Japan). Transduodenal and trans gastric punctures were performed according to the tumor location, with an average of 2 passes with 22 G needle (Interval: 1-3 passes) and average of two biopsies per tumor.

Samples obtained from the 20 cases were analyzed in the anatomopathology laboratory. The analyses performed were: Ki-67 index (K2 Leica Microsystems, Barcelona, Spain). The number of nuclear-stained tumor cells counted was >500 cells in each section under 400-fold magnification Immunocytochemistry (**ICQ**) for chromogranin, synaptophysin, neurospecific enolase (**ENE**), CD 56 and various hormones or peptides (gastrin, insulin, etc.), in case of suspicion of functioning tumor was performed.

There was surgical confirmation (gold standard) in 50% of cases. Immunohistochemistry (**IHC**) and Immunohistochemical staining for Ki67 were also performed in the surgical specimen and the Ki67 labeling indices were determined by digital image analysis.

An expert cytologist was present in the examination room to check the cellularity in the sample obtained (**ROSE**) and to diagnose the compatible PNET.

Statistical Analysis

The sensitivity and accuracy of Ki-67 index and the ICQ tests performed in the samples obtained by EUS-FNA with 22 G needles were analyzed. Diagnostic sensitivity was obtained using the formula: TP / (TP + FN), where TP and FN mean true positive and false negative,

respectively, obtained from the biopsies performed. The confidence interval of 95% of sensitivity was calculated using the exact -Pearson-Klopper method. The level of significance (statistically significant: $p < 0.05$) using Fisher's exact statistical test and the Pearson Chi-square test.

RESULTS

Patients selected for the final analysis (n= 20/28) were: 10 women (50%) and 10 men (50%) with a mean aged of 56 years; Interval: 30-71 years.

Location and number of tumors of cases studied: head n=6 (30%), uncinate n=2 (10%), body n=5 (25%), tail n=6 (30%), head-body-tail n=1 (5%) with multiple endocrine neoplasia (**MEN**).

The mean lesion size was 24.5 mm (Interval: 10-60 mm). Most of tumors were non-functioning n= 18 (90%). In four of the patients, tumors were MEN-1 type (4/20, 20%) (2 of them non-functioning).

In 90% of samples obtained, the ICQ study was performed (18/20) and in two cases there was not enough material for the assessment (10%).

In 80% of cases (16/20), the Ki-67 index was quantified and in four cases the sample obtained was not sufficient to evaluate the Ki-67 index (20%) (4/20), where two of them had a size lower than 2cm. The confidence interval (**CI**) was 95% (56% -94).

Ten patients underwent surgery, 5 women and 5 men. Of the samples obtained, the ICQ, IHC and the Ki-67 index were positive in 100 % of cases, with a correlation of 80% between the sample obtained using EUS-FNA and the surgery.

The sensitivity of EUS-FNA for diagnosis and classification of pancreatic neuroendocrine tumors, according to the WHO 2010 classification, in our study was of 80% (16/20).

The degree classification of tumor, according to the WHO 2010 scale, was maintained in all cases except in two patients (2/20, 10%). One of them (5%) was relabeled from G1 to G2 and another (5%) from G2 to G3, after receiving chemo radiotherapy (**CRT**). These two cases, as well as another patient classified as G3, currently have a 2-year survival and have hepatic metastases. The 17 patients classified as G1 and G2 are currently alive and metastases-free (17/20, 85%).

The distribution of patients, according to the final classification was: G3: 2 cases (10%), G2: 6 cases (30%) and G1: 12 cases (60%). There were no major complications attributable to EUS-FNA.

DISCUSSION

The new WHO 2010 classification of TNEPs, which classifies them into well differentiated tumors (grade 1, G1 and grade 2, G2) and poorly differentiated tumors (grade 3, G3), based on the ki-67 labeling index and mitotic count. This fact makes this biomarker essential for the tumor classification and the assessment for prognosis and patients survival. Therefore, methods for

obtaining tissue suspicious of PNET should be adequate so that the extracted material be optimal for such analysis.

The EUS-FNA with 22G needles was the chosen method to obtain the samples, due their safety and little infrastructure needed to be carried out. In addition, the diagnostic sensitivity of EUS-FNA is > 90% and the material obtained is suitable for performing ICQ techniques, which substantially improves diagnostic sensitivity. From the results obtained using EUS-FNA with 22G and by comparing our results with those found in the literature, we found that these were similar.

In 2014, Unno J. et al, published a study performed with 22G needle in SETs of 35 patients, achieving a yield for ICQ of 92.1% and a concordance of Ki-67 index of 89.5%[12]. Sugimoto M et al., one year later, published a study with 19, 22 and 25G needles, in which 22G were used in 6 of the 8 operated patients, obtaining a concordance of Ki-67 index of 87.5% [13]. In our series of 8 patients that underwent surgery, we obtained a profitability of 90% for ICQ and 80% for Ki-67 index, with a correlation of 80%.

The first study by Larghi et al. [14], published in 2012, on 28 cases of non-functioning PNET was performed with EUS-FNA 19G needles and therefore, with a higher potential risk, they obtained adequate samples for histological analysis in 93.3% (28/30). In our series the samples obtained were adequate for the ICQ study in 90% of the cases (18/20). They were able to evaluate Ki-67 in 92.9% of cases (26/28).

Carlinfante et al. [15], in their study published in 2014, were able to evaluate Ki-67 in 87 % of 53 NET, with a sensitivity of 92%. In our series with 22 G needles, the yield was 80% (16/20).

In the study by Larghi et al. [14], 12 patients underwent surgery (42.85%) and the preoperative and postoperative analyzes were concordant in 83.3%; 2 patients were reclassified from G1 to G2 or from G2 to G1 respectively. In our series, patients undergoing surgery were 10 (50%) and the concordance of results before and after surgery was 80% (8/10), only a single case being changed from G2 to G3, after CRT, and another from G1 to G2 after surgery.

Fujimori N. et al. [16], in their work of 2016 evaluating the efficacy of endoscopic ultrasound and EUS-FNA for the diagnosis and classification of pancreatic neuroendocrine tumors according to WHO-2010 classification, EUS showed a significantly higher sensitivity to identify PNETs (96.7%) than CT (85.2%), MRI (70.2%) and ultrasonography (75.5%). The sensitivity of USE-FNA for diagnosis of PNET was 89.2% (Table 2).

Table 2: Comparison of our series with Larghi et al series [14].

	Larghi et al.	Our series
N° de cases	28	20
Needle used	19G	22G
Samples suitable for ICQ	93,3% (28/30)	90% (18/20)
Evaluation of the ki-67 index	92,9% (26/28)	80% (16/20)
Patients undergoing surgery	42,85% (n=12)	50% (n=10)
Concordant results after surgery	83.3% (10/12)	80% (8/10)
Reclassification after surgery	G1 a G2 (n=1)	G1 a G2 (n=1)
	G2 a G1 (n=1)	G2 a G3 (n=1)

ICQ: Immunocytochemistry.

Further studies analyze factors that may affect the quality of samples by comparison the results of EUS-FNA with 19G (3 cases), 22G (46 cases) and 25G (4 cases) needles, obtaining concordances with surgery > 80%, finding no significant differences. The small number of cases for 19G and 25G needles makes difficult to reach a firm conclusion and would be necessary more studies with greater number of cases [17]. Other review studies have also found no significant differences in relation to the size of the needle used.

Needles of 25G might have a slightly greater sensitivity and adequacy than 22G needles, but provide no advantages with respect to precision, number of passes or complications. Needle selection is a complex process and will depend ultimately on the morphology of the lesion, the location of the tumor and the availability of a cytopathologist during of sampling [18].

According to some authors [19], the diagnostic yield of Ki-67 index obtained by USE-FNA, in addition to the size of the needle, could be related to the size and localization of PNET, a fact that we have not observed in this study. However, to obtain results with greater consistency and to be able to perform a comparative study of the different types of needles, the number of samples should be higher in each of series.

Díaz C. et al., performed a retrospective review of all cases of EUSFNA cytology of pancreatic lesions performed between 2006 and 2016 in their hospital, with a similar number of cases analyzed (n=24) [20]. Among their results, the mean age was 56.8 years and most patients were males (54%), almost the same data of patients of our series, mean 56 years; Interval: 30-71 years, but with no differences between males and females (50%). The location of their tumors was mainly in head and tail as in our series; with the same average we found (around 30%). The average tumor size (36 mm) was higher than in our series (24.5 mm), as well as the mortality of patients with G1 tumors (22%) that was 0% in our patients with the same graded tumor, probably, related to size.

The greatest relevance of EUS-FNA procedure for PNETs is the ability to obtain, without surgery, adequate samples to grade the tumor according to WHO 2010 criteria. This may give

doctors the possibility to decide the best option to obtain a successful outcome for patients. That is why most of studies are focused on the comparison of Ki-67 index obtained in EUS-FNA specimens and in resected specimens as the gold standard criterion, with the aim to find the best procedure to obtain the higher concordance with grading of samples obtained by surgery. Interesting examples are the study of Weynand B et al. [21], Hasegawa T et al. [22], He SR et al. [23], Barnes J et al. [24], Boutsen L et al. [25], where researchers agree about the good concordance of results when the cell count is > 2000 [23] and its systematically inclusion in the clinical protocols [21,23]. However they also recommend being cautious with grade 2 (**G2**) tumors, where cytology can underestimate grading owing to tumor heterogeneity [21]. Low Ki-67 index has not to be the only parameter to choose a more conservative treatment approach if there is clinical, biochemical or radiological evidence suggestive of a more aggressive disease pathology [24]. Regarding to our study, t can be concluded that the EUS-FNA to obtain tissue of PNET with 22 G needles seems to be a feasible, safe and effective procedure to determine the definitive anatomopathological diagnosis of this type of tumor, since the samples obtained were sufficient to establish the diagnosis in 80% of cases. It has a high diagnostic sensitivity for the cytological confirmation and a high accuracy in the evaluation of the Ki-67 index. However, to reach higher statistical significance and determine if the pre-operative Ki-67 index obtained by USE-FNA with 22G needles can accurately evaluate the prognosis of PNETs, more prospective comparative studies are needed with larger number of patients and with different specialists carrying out the procedure.

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