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TOPIC HIGHLIGHT

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Endoscopic ultrasound guided fine needle aspiration and useful ancillary methods

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Abstract

The role of endoscopic ultrasound (EUS) in evaluating pancreatic pathology has been well documented from the beginning of its clinical use. High spatial resolution and the close proximity to the evaluated organs within the mediastinum and abdominal cavity allow detection of small focal lesions and precise tissue acquisition from suspected lesions within the reach of this method. Fine needle aspiration (FNA) is considered of additional value to EUS and is performed to obtain tissue diagnosis. Tissue acquisition from suspected lesions for cytological or histological analysis allows, not only the differentiation between malignant and non-malignant lesions, but, in most cases, also the accurate distinction between the various types of malignant lesions. It is well documented that the best results are achieved only if an adequate sample is obtained for further analysis, if the material is processed in an appropriate way, and if adequate ancillary methods are performed. This is a multi-step process and could be quite a challenge in some cases. In this article, we discuss the technical aspects of tissue acquisition by EUS-guided-FNA (EUS-FNA), as well as the role of an on-site cytopathologist, various means of specimen processing, and the selection of the appropriate ancillary method for providing an accurate tissue diagnosis and maximizing the yield of this method. The main goal of this review is to alert endosonographers, not only to the different possibilities of tissue acquisition, namely EUS-FNA, but also to bring to their attention the importance of proper sample processing in the evaluation of various lesions in the gastrointestinal tract and other accessible organs. All aspects of tissue acquisition (needles, suction, use of stylet, complications, *etc*.) have been well discussed lately. Adequate tissue samples enable comprehensive diagnoses, which answer the main clinical questions, thus enabling targeted therapy.

Key words: Endoscopic ultrasound; Endoscopic ultrasound-guided fine needle aspiration; Endoscopic ultrasound-guided tissue acquisition; Fine needle aspiration cytology; Ancillary studies; Molecular testing; Flow cytometry immunophenotyping

Core tip: All aspects of endoscopic ultrasound tissue acquisition have been well discussed in recent studies. In addition to discussions about important factors that impact tissue acquisition, in this article we have highlighted the methods of ancillary testing needed to satisfy the growing demands of precision medicine standards. Adequate tissue samples and appropriate ancillary testing enable comprehensive diagnosis, and answer the main clinical questions, thus enabling targeted therapy.

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INTRODUCTION

Endoscopic ultrasound guided fine needle aspiration (EUS-FNA) is an effective and widespread method for the procurement of pancreatic tissue and, as such, is capable of providing an accurate preoperative diagnosis. EUS-FNA is also the method of choice for the evaluation of mural lesions of the gastrointestinal tract and deep seated structures, such as mediastinal lesions, lymph nodes, liver, spleen and suprarenal glands. It has become very important to accurately determine the type of tumor, to precisely differentiate between primary tumors and metastases, and to identify potentially actionable genetic mutations in order to tailor an adequate therapeutic approach. The novel treatment protocols and application of chemotherapeutic agents are often based on the molecular markers present in the tumor tissue, where classic cytological or histological analyses fail to provide necessary information for further treatment decisions, especially important for neo-adjuvant chemotherapy. Therefore, there is a growing demand to obtain sufficient amounts of tissue samples from suspected lesions for further analyses, or for the procurement of core tissue for histological analysis, if necessary.

TECHNICAL ASPECTS OF EUS-GUIDED TISSUE ACQUISITION

Needles

Several types of needles for EUS-FNA are present on the market, and there is an abundance of recently published high quality references addressing this important issue. EUS-FNA can be performed using 25-gauge (G), 22-G or 19-G needles. Similar diagnostic yields are reported for all types of needles[1-6]. The most widely used needle for EUS-FNA is the 22-G needle. Thinner and more flexible, the 25-G needle should have better performance for targeting the uncinate process and for the transduodenal approach to the pancreatic head. Still, no significant difference in the diagnostic accuracy has been described[6-8]. The flexibility of the needle is especially important in locations where the scope needs to be bent to target the lesion. However, only one study of 24 patients documented a better technical success rate of the 25-G needle but the study evaluated only lesions located in the uncinate process[3].

On the other hand, a randomized controlled study of 131 patients did not show a significant difference between the 22-G and the 25-G needle in the diagnostic yield, the ease of targeting the lesion, malfunction of the needle, and the complication rate[2]. A larger 19-G needle was developed to obtain larger amounts of material from the lesions, but compared to the 22-G needle, the 19-G needle is stiffer and has a higher rate of technical failure. This is seen in particular in pancreatic head lesions[3]. There is one study showing that the 19-G needle has a higher diagnostic accuracy than the 22-G needle, but technical failures were excluded from the calculations[3]. In a multicenter study with the novel 19-G flexible needle made of nitinol (Expect 19 G Flex, Boston Scientific, Natick, MA, United States), there was no significant difference in diagnostic accuracy between the 22-G and the 19-G needle, but histological core tissue was obtained in a larger number of patients using the 19-G flexible needle[9]. A needle especially designed to obtain core tissue for histological analysis was the Trucut (Cook Endoscopy, Winston Salem, NC, United States) biopsy needle. The idea about the needle was good, but the needles had weak performance due to technical design problems[10-12]. The ProCore (ProCore, Cook Endoscopy) needle overcame some obvious limitations of the Trucut needle, enabling easier acquisition of the core tissue for histological analysis due to the special design. However, transduodenal passage still remained a challenge[13-15]. In the ProCore gamma, there are also 22-G and the 25-G needles with the same design available. Two groups of authors recently studied the utility of the standard 19-G needle[16] and the 19-G flexible needle[17] for obtaining core tissue for histological analysis. The standard 19-G needle experienced problems with the transduodenal approach, while the 19-G flexible needle had no technical difficulties in the acquisition of the specimens for histological analysis[17].

Stylet

The role of stylets should be to prevent sample contamination by cells that do not originate from the targeted lesion. However, three different studies have not found any advantage or disadvantage of using stylets for EUS-FNA[18-20]. The procedures in which a stylet is used may be clumsy and time-consuming. Although some authors advocate no stylet FNAs, we find it very useful for contamination prevention in routine practice. Furthermore, the stylet can be very useful for pushing out the sample from the needle to the slides or into the liquid transport medium.

Suction

Suction applied during EUS-FNA will result in acquisition of more material for further analysis, but it will also make the specimen bloodier[21,22], thus potentially hindering the morphological tissue analysis. EUS-FNA without suction provides specimens with less blood. Applying suction during EUS-FNA of solid lesions was associated with a significantly higher sensitivity (86% *vs* 67%; $P = 0.005$) in one study only[21]. Another study reported establishing diagnoses in over 90% of patients without applying suction[22]. For lymph node sampling, applying suction can result in significantly bloodier samples which

can affect the diagnostic yield of EUS-FNA[23].

Tissue acquisition

Obtaining "diagnostic" tissue by EUS-FNA may be hindered by necrosis of the suspected lesion. Obtaining the tissue from the peripheral area of the lesion or from multiple areas in the "fanning" way may improve diagnostic yield. A sufficient number of passes must be performed to provide enough material for analysis, and in the case of failure, the procedure needs to be repeated[24]. One study described the advantages of the "fanning" technique in primary tumors, although these advantages were not described regarding lymph node sampling[25].

COMPLICATION

Tissue acquisition by endoscopic ultrasound is a safe procedure. The reported overall complication rate ranges from 0.3% to 2.2%. The most worrisome complication of an FNA is tumor cell seeding. However, only three cases of needle tract seeding following EUS-FNA have been reported to date[26]. The major complications of EUS tissue acquisition are infections of cystic lesions, bleeding and acute pancreatitis[27,28]. In order to prevent infection, prophylactic antibiotics must be considered for EUS-FNA of cystic lesions. Intracystic bleeding is rarely significant. A change in echogenicity of the cystic fluid indicates intracystic bleeding. The bleeding usually resolves spontaneously, but some medications that affect the process of coagulation may pose problems. In order to avoid bleeding, clopidogrel must be discontinued 7 d prior to the procedure, low molecular-weight heparin 12-24 h prior, and unfractionated heparin 6 h prior to EUS-FNA. Aspirin does not have to be discontinued. It is recommended to discontinue warfarin 5 d prior to the procedure and bridge it with heparin to avoid thrombotic events in high risk patients[29]. Coagulation parameters should be checked before the procedure.

ON-SITE CYTOPATHOLOGIST

Rapid on-site evaluation (ROSE) by attending cytopathologists undoubtedly improves the diagnostic yield of EUS-FNA, reduces costs and decrease the number of repeated procedures[30]. Macroscopic specimen analysis is of limited value when performed by an endosonographer, but it may be useful for the rough assessment of specimen adequacy. When performed by an experienced cytopathologist[24,31], it can save time and reduce the number of passes necessary to acquire additional tissue after the initial ROSE. The ROSE findings and clinical suspicions should be considered as the starting point for the selection of further ancillary methods.

ANCILLARY METHODS

Individualized medicine and various options of targeted therapy in modern health care increase the demands for molecular and other ancillary testing on small tissue specimens. Requests for minimally invasive procedures for tissue acquisition are especially emphasized in patients with unresectable malignant diseases, and for patients requiring neoadjuvant chemotherapy. EUS-FNA is an efficient, cost-effective and minimally invasive method for tissue acquisition with diagnostic accuracy comparable to excisional biopsy[32].

A number of ancillary laboratory tests are routinely employed in establishing diagnostic and prognostic factors in tissue specimens from various lesions of the gastrointestinal tract, pancreatic masses and other accessible organs. These include microbiology, immunocyto(histo) chemistry, flow cytometry, biochemical analyses, conventional cytogenetics and various molecular methods.

The correct choice of ancillary method often depends on the type of aspirated sample, the quality of the specimen and the preliminary cytological diagnosis (ROSE). It is also institution- and resource-dependent, hence, in-house protocols and algorithms defining sample processing should be developed. The on-site cytopathologist can serve to liaise between the laboratory and the clinician, triage the samples and ensure optimal sample collection and processing[33]. This approach is gaining in importance as we witness the expanding role of cytological samples in providing data for targeted therapy[34].

Except in the formalin-fixed, paraffin-embedded (FFPE) histological tissue, a number of ancillary tests are also easily performed on the EUS-guided FNA samples[27]. Specimens can be processed in different ways depending on the laboratory set-up and the pathologist's preference.

Most laboratories prefer at least two direct cytological smears for ROSE, one air-dried and one ethanol-fixed, then rinsing of the needle for the preparation of cell blocks (FFPE tissue). Another method of cell block preparation is simply letting the aspirated sample clot on the glass and then fixing it in formalin as described by Bellizzi and Stelow[35]. Different practices, with more direct slides made on the spot are not unusual in institutions where a cytopathologist is present on-site to make the decision regarding specimen processing. The advantages and disadvantages of cell block preparation, and its use, are discussed elsewhere[33,36].

Core tissue for histological diagnosis is not easily obtained from the pancreas and other deep-seated sites, as adequate tissue sampling requires special types of needles. Reported sensitivities and specificities are similar for FNA and core biopsy for malignant diagnoses[13]. In addition, formalin, as a fixative, can damage DNA for molecular testing. Thus, EUS-FNA remains the primary means of establishing a tissue diagnosis preoperatively, and it ensures samples for ancillary testing.

A major complaint regarding EUS-FNA is that it does not yield enough tissue to meet the increasing demands for ancillary (molecular) testing[33]. This drawback can be overcome by implementing ROSE, which determines the number of passes necessary to ensure adequate sample

Table 1 Possible methods of sample processing

Sample types	Ancillary methods
Direct cytological smears	Cytomorphological diagnostic evaluation
	ICC staining
	Molecular testing (FISH)
	Excellent source for isolation of good quality DNA[39,40]
Cell blocks (formalin-fixed, paraffin-embedded tissue)	IHC
	Various molecular tests
	Tissue storage
Touch imprint slides (tumor cell rich slides made by imprinting the tumor)	IHC
	Molecular testing (FISH)
Fresh aspirated specimens (especially of lymph nodes)	Flow cytometry - immunophenotyping
	RNA based gene expression studies
	DNA based studies[41]
Fresh frozen (cryopreserved) FNA specimens	RNA based gene expression studies
	DNA based studies[42]
FTA cards (filter paper)	Effective RNA and DNA preservation
	Not suitable for morphology[43]
Formalin fixed paraffin embedded core tissue	Histological morphologic evaluation
	IHC and molecular studies

FISH: Fluorescence in situ hybridization; FNA: Fine needle aspiration; ICC: Immunocytochemistry; IHC: Immunohistochemistry.

collection, processing and storage[37]. EUS-FNA has some additional advantages, including sampling of the wider area of the lesion. Aspirates also usually yield more tumor and less stromal cells than core biopsy[33]. The inability to assess tissue architecture, and to determine some valuable prognostic factors, such as the number of mitoses or angioinvasion, remains a major disadvantage. The assessment of such prognostic factors can be a problem with small core biopsy specimens as well.

Based on the type of specimen, some tests are more likely to be employed then others. Liquid samples from cystic pancreatic lesions need to be evaluated macroscopically for viscosity, which can provide valuable information about the nature of the cyst. The cyst fluid is then submitted for routine cytology, biochemical analysis, microbiology if necessary, and possibly for molecular analysis[38]. If the fluid is thick, direct smears, instead of cytospins, can be made for cytomorphologic evaluation.

For solid lesions, immunocytochemistry (ICC) or immunohistochemistry (IHC) are methods of choice for the diagnosis and tumor subclassification. Cell blocks or smears can also be submitted for molecular tests if needed. Lymphoid tissue and liquid specimens are highly suitable for the flow cytometry immunophenotyping.

There are several possible means of processing aspirated samples obtained by EUS-FNA for molecular and other ancillary tests (Table 1).

BIOCHEMISTRY

The most useful addition to cytology in routine preoperative evaluation of pancreatic cystic lesions is the biochemical measurement of enzyme levels, especially carcinoembryonic antigen (CEA) and amylase levels[44].

The cytological analysis of cystic lesions is accurate in diagnosing cystic pancreatic neoplasms and is currently the only method that can accurately differentiate between malignant and benign cysts, as well as between various types of benign cysts. The diagnostic yield of cytology alone is limited because, despite a high specificity, it has low sensitivity for detecting malignancy due to the low cellularity of cystic lesions[45]. The measurements of CEA and amylase levels in the cystic fluid help differentiate serous from mucinous cysts[46]. Mucinous cysts are considered, at least, premalignant or low-grade non-invasive neoplasms, but the distinction of mucinous cystic neoplasms (MCN) from non-mucinous cysts is not sufficient to determine the need for operative treatment, as it was a few years ago[47]. Cysts with a low risk of progression are suitable for surveillance. The threshold value of 192 ng/mL is usually found to be distinctive between mucinous and non-mucinous cysts. CEA levels have limited value in the subclassification of mucinous cysts[48,49], and contrary to early studies[50], subsequent studies[51,52] have shown that CEA levels do not discriminate between malignant and benign mucinous cysts.

Amylase levels are usually higher in post-pancreatitis lesions, *e.g.*, pseudocysts, but can be elevated in mucinous cystic neoplasm as well. Al- Rushdan[49] reports that the measurements of amylase levels do not discriminate between the various types of MCN and intraductal papillary mucinous neoplasms. Nevertheless, cytology, coupled with biochemical measurements of CEA and amylase levels in cystic fluid, is currently the best way of assessing pancreatic cysts preoperatively. The addition of molecular studies seems promising[53,54].

IMMUNOCYTOCHEMISTRY AND IMMUNOHISTOCHEMISTRY

A wide application of ICC and IHC on small tissue samples enables better characterization and subclassification of tumors, which have become increasingly important with regard to targeted therapy for malignant diseases.

The main principle of these methods is detecting and visualizing antigens on the cell surface, cytoplasm or nucleus. The method is suitable for FFPE samples, including excisional or small core biopsies or cell blocks obtained during EUS-FNA. It is also routinely performed on air-dried, previously unstained direct cytological smears, cytospins or liquid based cytology preparations. ICC can also be performed on previously stained (Papanicolaou) and archived cytological smears.

Presently, IHC (ICC) is routinely used for the diagnosis and subtyping of various tumors, and is increasingly used for the evaluation of prognostic factors[55,56].

While the diagnosis of pancreatic ductal adenocarcinoma does not require IHC confirmation, other solid neoplasms, including lymphoma, neuroendocrine tumors (NET), solid pseudo-papillary neoplasms or acinic cell carcinoma, need IHC or ICC for the final diagnosis. The biological behavior of pancreatic NETs cannot be predicted by morphology alone, so proliferative markers, such as Ki-67, have become an essential tool for grading pancreatic NETs, which is necessary for adequate patient management. Spindle cell submucosal gastric tumors are another example of neoplasms which require IHC or ICC for definitive diagnosis and grading.

Most laboratories use direct smears for ROSE, and cell blocks for IHC and other ancillary methods. Major disadvantages of cell blocks include low cellularity, pooled nature of the specimen, uneven distribution of tumor cells in the block, and the lack of ROSE. Because of these drawbacks, we should bear in mind that direct smears and cytospins are also suitable for ICC.

FLOW CYTOMETRY IMMUNOPHENOTYPING

Flow cytometry is a technology that simultaneously measures and then analyses multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a laser-beam. The measured properties include particle relative size, relative granularity or internal complexity, and relative fluorescence intensity.

Immunophenotyping has become a fundamental step in the diagnosis and subclassification of hematologic malignancies, especially nodal and extra nodal lymphoproliferative disorders. The main advantage of the method is the possibility of detecting a specific surface or cytoplasmatic antigen on targeted cells by applying a complete panel of antibodies to small samples. Other applications include DNA/RNA analysis and functional analysis (oxygenation, enzyme activity, phagocytosis, *etc*.).

EUS-FNA coupled with flow cytometry has become widely used in evaluating the deep-seated lesions, especially mediastinal and retroperitoneal lymph nodes[57], and there are several described cases of a primary lymphoma of the pancreas diagnosed by EUS- FNA and flow cytometry[58-60]. Flow cytometry has broadened the usefulness of EUS-FNA and increased its diagnostic yield[55].

CONVENTIONAL CYTOGENETICS

Conventional cytogenetics for detecting structural chromosomal abnormalities studying the whole karyotype is not routinely used on EUS-FNA samples of pancreatic lesions, but is used in the diagnostic algorithms of some deep-seated soft tissue lesions and hematological malignancies. EUS-FNA can be a good tissue source for cell cultures when needed[61].

MOLECULAR METHODS

With emerging targeted therapies for various types of malignant diseases, there is an increased demand for molecular testing as part of the routine diagnostic and prognostic workup, as well as for research purposes. The main goal is to identify any actionable genetic mutations. EUS-FNA is a minimally invasive, highly suitable method of tissue acquisition for molecular studies. The integrity of DNA and RNA available from FFPE tissue is often compromised because of the methods for tissue fixation and storage. Although genomic studies of large archival FFPE cohorts are critical for molecular studies, the best material for molecular studies remains fresh tissue[62]. With improved isolation techniques, DNA isolation is possible from paraffin-embedded tissue. Cytological material obtained by EUS-FNA provides good quality DNA regardless of the differences in sample preparation[63]. This is partly due to the use of nucleic acid-friendly fixatives other than formalin[34]. FNA samples for molecular studies can be processed in different ways[33], depending on the type of the sampled tissue (ROSE diagnosis), the laboratory resources, and the type of test needed. The choice of ancillary method should be focused on answering the dominant clinical questions.

Various methods of molecular analysis are employed in the evaluation of pancreatic cysts and solid masses. The ones most frequently used in diagnostic and prognostic workups are the polymerase chain reaction (PCR) based techniques and fluorescence *in situ* hybridization (FISH).

PCR-based DNA studies usually evaluate the *kras* mutational status and the loss of heterozygosity of several markers situated on different chromosomes, particularly in the cystic pancreatic lesions, in an attempt to increase the diagnostic sensitivity[64,65]. After the initial enthusiasm, recent studies have reported low sensitivity for the *kras* mutational analysis in cystic fluid[66]. The value of the *kras* analysis in solid pancreatic masses is not as well documented, as there are only a limited number of studies addressing the issue, and they have reported variable results[67-69]. Salek *et al*[70] Studied the *kras* mutation, p53 and allelic loses at 9p and 18q, and have concluded that they are not suitable prognostic markers for pancreatic cancer. Nevertheless, the meta-analysis by Fuccio *et al*[71] concluded that *kras* mutation analysis can be helpful in the diagnostic workup of pancreatic lesions when there is a limited tissue sample obtained and the diagnosis is inconclusive. A study by Pellisé *et al*[72] showed increased

sensitivity to the detection of lymph node micrometastases using hypermethylation analysis performed by methylation-specific PCR using EUS-FNA samples from the lymph nodes of patients with various types of gastric, intestinal and lung cancer.

RNA studies of gene expressions are presently limited to research purposes, but there are several reports analyzing RNA expression of various genes for diagnostic expression analysis[73,74], and a report studying the preservation of the sensitive RNA molecules for future use[42]. FISH is a molecular method performed on the intact cell nuclei and has the advantage of visualization of the cells with the detected abnormality. There have been several attempts to use FISH in the diagnostic workup of pancreatic lesions using probes for different markers (chromosomal polysomy, deletions, *etc*.). Cohorts of included patients were usually small, so a comparison of the data is of limited value. The main conclusion is, that in the setting of inconclusive cytology, FISH can aid in the diagnosis of pancreatic malignancy[64,75,76]. On the other hand, FISH is crucial for the diagnosis and prognosis of some tumors with well-defined chromosomal abnormalities and gene rearrangements, such as lung cancers (including metastases), lymphomas and other tumors. As there are no specific molecular markers identified as yet for the specific diagnosis of solid or cystic pancreatic masses, the research area remains wide. High-throughput techniques look promising in finding adequate biomarkers for pancreatic neoplasms. Methods using DNA microarrays, comparative genomic hybridization and DNA sequencing can be performed on samples obtained by EUS-FNA, as high quality DNA can be harvested and adequately processed[77].

CONCLUSION

The main issue with tissue acquisition is obtaining a large enough sample that will be able to provide a comprehensive diagnosis to satisfy the growing demands for individualized patient management and targeted therapy for the malignant disease. EUS-FNA remains a minimally invasive, easy to perform, and altogether more effective method of sampling tissue from deep-seated lesions than any other available method. Numerous factors influence the yield of the EUS-FNA. At present, the best results are achieved by collaboration between a skilled endosonographer performing the EUS-FNA (tissue acquisition) and an on-site cytopathologist. The choice of the needle and sampling technique should be up to the performing endosonographer. The preliminary cytological diagnosis directs further action. The ROSE findings and clinical suspicions should be taken as the starting point for the selection of further ancillary methods. The choice of specimen processing should be in accordance with institutional protocols and available resources in the best interest of optimal patient care.

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