Molecules and markers for endosonographers: What do we need to know and measure?

M. Al-Haddad, M. B. Wallace
Division of Gastroentorology and Hepatology, Mayo Clinic College of Medicine, Jacksonville, FL

Introduction

Endoscopic ultrasound (EUS) has become an indispensable tool for the diagnosis and staging for gastrointestinal and thoracic malignancies. Thanks to the safety and reliability of fine needle aspiration (FNA), this has become the method of choice for tissue acquisition from lymph nodes and tumors in various locations within the thoracic and abdominal cavities.

In the recent years, EUS-FNA has offered a better understanding of the pathophysiology of cancer. Similar to the adenoma-carcinoma sequence, other cancers including pancreas, lung, and esophagus may follow a comparable multi-step process of carcinogenesis. A necessary component of these is the ability to go "beyond cytology" to use fine needle aspirates to characterize the genomic and proteomic profiles of cancer and precancerous tissue.

Molecular characterization of FNA samples: The basic concepts

Although DNA is relatively stable in excised tissue, proteins and particularly RNA are unstable and must be handled in special ways to preserve them for characterization. The first role of the endoscopist is to obtain and preserve tissue suitable for further molecular characterization. In the first part of this review, we will focus on these initial steps. The methods for genomic and proteomic analyses are beyond the scope of this article and interested readers are referred to recent reviews [1,2].

RNA destroying-enzymes (RNAase enzymes) are very ubiquitous in the environment. Therefore, it is imperative to prevent rapid degradation of RNA in order to preserve fine needle aspirates for genomic studies. The simplest method to do this is through the use of RNAase inhibitor solutions such as RNA-later (Quagen Inc. Valencia Ca, USA) or RNA Stat 60 (RNA STAT-60®; TEL-TEST, Friendswood, TX, USA). The needle aspirate can be quickly mixed with these reagents. In our laboratory, we immediately place the FNA sample into an eppendorf tube in a wet ice bath, centrifuge in a small desktop device in the EUS room (1000 RPM at 4 deg. C, 1 minute), remove the supernatant, and resuspend the cell pellet in RNA stat 60. If RNA stabilization is not available on-site, the aspirate can also be snap frozen in liquid nitrogen and stored (preferably at –70 to –80C) for later RNA stabilization. Every effort should be made to handle the specimen in an RNAase-free environment including clean gloves and a surface that has been cleaned with an RNAase inhibitor solution.

In the remainder part of this review, we will focus on organ-specific applications of the biological markers. This will include lesions of the pancreas and gastrointestinal stromal tumors (GIST). The use of biomarkers in staging non-small call lung cancer will be discussed in a separate review.

Pancreatic lesions

Diagnosis of early pancreatic cancer remains a challenge mainly due to the lack of a standard screening test. Therefore, improving long-term survival relies primarily on detecting and treating early pancreatic cancer and thus reducing the incidence of pancreatic cancer.

EUS is increasingly considered a valuable tool for the evaluation of the pancreatic cystic and solid lesions. It offers the highest resolution available for imaging the entire pancreatic parenchyma and duct. EUS provides detailed information regarding the wall and septations of pancreatic cysts and shed some light on the underlying pathology of such cysts.

Cystic lesions of the pancreas are being detected with increasing frequency, yet remain poorly understood. They are broadly divided into mucinous and nonmucinous lesions. Mucinous lesions (mucinous cystic neoplasms and intraductal papillary mucinous neoplasms (IPMNs)) are considered to be premalignant or malignant and surgical resection is generally recommended for appropriate candidates although it is increasing recognized that some IPMN lesions have a very low rate of malignant degeneration and may not require resection [3]. Non-mucinous lesions (serous cystadenomas and pseudocysts) have low or no malignant potential. Resection is only indicated in the setting of uncontrollable symptoms. Accurate methods to diagnose and determine the malignant potential of cystic lesions of the pancreas are highly desirable but current methods are limited. Currently, the only definite way to identify such lesions reliably is to surgically resect the part of the pancreas containing the suspicious lesions and examine them histopathologically.

EUS-guided fine needle aspiration (EUS-FNA) and cyst fluid analysis has been valuable to differentiate cystic lesions of the pancreas. As cytology of cyst fluid can be non-diagnostic due to low cellularity of the aspirated fluid, investigators have evaluated the use of tumor markers in the cyst fluid [4,5]. CEA and CA 72–4 were found in high concentrations in mucinous cystic neoplasms. Various series have demonstrated that the cyst fluid CEA of benign cystic lesions is low [6,7]. The mean values ranged from 1.1 to 7.5 ng/mL in psuedocysts and serous cystadenomas.

The Cooperative Pancreatic Cyst Study evaluated the cystic fluid of 341 patients who underwent EUS-FNA of cystic lesions of the pancreas. Among these patients, 112 patients had surgical confirmation of the diagnosis. The receiver operator characteristic (ROC) curves were plotted for each tumor marker (CEA, CA 72–4, CA 125, CA 19–9, CA 15–3) to predict a mucinous or nonmu-

Correspondence: Michael B. Wallace, M.D. · Associate Professor of Medicine · Division of Gastroenterology and Hepatology · Mayo Clinic Jacksonville · 4500 San Pablo Rd · Jacksonville, FL 32224 · E-mail: wallace.michael@mayo.edu

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cinous cystic lesion. The accuracy was greatest for CEA and CA 72–4. The CEA cutoff value of 192 ng/mL provided the greatest accuracy of 0.79 (1.0 is perfect) with moderate sensitivity of 0.73 and specificity of 0.84. For CA 72–4, the cutoff value of 7 ng/mL provided an accuracy of 0.72, sensitivity of 0.80 and a specificity of 0.61. This study confirmed that mucinous cystic lesions have a much higher concentration of CEA (mean CEA-5607 ng/mL) as compared to nonmucinous cystic lesions (mean CEA-284 ng/mL) [8].

Microscopic premalignant lesions of the pancreas (e.g., IPMNs) are unlikely to be detected by serum markers given their noninvasive nature. No standard serum marker exists for screening of precancerous lesions, although these lesions pose the greatest risk for future invasiveness and, at the same time, have the highest chances of cure. It is projected that many of the underlying genetic abnormalities found in pancreatic ductal adenocarcinoma are found in such pre-cancerous lesions, although at lower prevalence. Therefore, some markers that are being investigated for pancreatic cancer diagnosis could potentially also predict the presence of these precancerous lesions [9].

Pancreatic juice analysis is being evaluated as a source of markers of pancreatic neoplasia [10], similar to other biomarker screening strategies (such as stool markers for colorectal neoplasia). Pancreatic juice can be collected during routine upper GI endoscopy after secretin stimulation. During EUS, fluid obtained from FNA of solid or cystic lesions represents an excellent source from which such markers can be obtained. This can be of particular importance in patients with diffuse and nonfocal changes of the pancreas as well as patients with a strong family history of pancreatic cancer. Pancreatic fluid contains higher concentrations of genomic and proteomic material allowing it to be a better substrate for further assays targeting screening for pancreatic cancer. For example, mutated *K-ras*, mutated *p53* [11,12], and telomerase [13,14], are more often detected in pancreatic secretions than in other sites such as plasma, duodenal fluid, or stool.

Mutant *K-ras* has been evaluated and found to be present in approximately 90% of pancreatic ductal adenocarcinomas. It can be easily detected using molecular assays and its presence has been shown to indicate worse prognosis [15]. However, *K-ras* mutations lack the specificity for pancreatic cancer; they were found to occur in smokers, patients with chronic pancreatitis, and pancreatic cystic lesions without cancer [16–18]. Mutant *K-ras* can also be detected in the blood of patients with advanced pancreatic cancer [12,19]. Despite this limitation, a sensitive assay has been recently developed that can quantify levels of mutant *K-ras* in blood and pancreatic juice. Further research is needed though to prove that quantifying mutant *K-ras* levels could improve the diagnostic utility of mutant K-ras [20].

TP53 gene mutations have been generally been investigated as a potentially specific diagnostic marker in a variety of cancers. Several technologically advanced assays have been developed (like single-strand conformational polymorphism) in an attempt to identify the entire spectrum of *TP53* gene mutations. When detected in pancreatic ductal adenocarcinoma, it generally occurs late in the neoplastic process and indicates invasivness. These mutations were found in approximately 70% of invasive cancers [21]. Although a few nucleotide hot spots of *TP53* gene mutation are known to exist, mutations occur throughout the gene [22]. In one study, investigators reported the presence of *TP53* gene mutations in pancreatic juice samples and in brush cytology specimens of 40%-50% of patients with pancreatic cancers [23]. Therefore, the detection of P53 mutations in pancreatic juice has the potential to become an applicable diagnostic strategy if improvements in mutation detection technology can enable accurate detection of such mutations.

The detection and quantification of aberrantly methylated DNA in pancreatic fluid can be a promising approach to the diagnosis of pancreatic cancer. A recent study by Matsubayashi et al evaluated the presence of DNA methylation alterations in the pancreatic juice of patients with suspected pancreatic disease [10]. The prevalence of pancreatic juice methylation in patients with chronic pancreatitis was less than in patients with pancreatic cancer but higher than in controls.

Finally, combining histological examination and immunoassays can significantly enhance diagnostic accuracy of pancreatic cancer. In a recent study from Japan, a total of 62 patients with solid pancreatic masses (51 pancreatic cancers and 11 chronic pancreatitis) underwent EUS-FNA [24]. Tissue specimens were evaluated by routine histological examination and p53 immunostaining. p53 protein overexpression was observed in 67% patients with pancreatic cancer, but not in patients with chronic pancreatitis.

Gastrointestinal Stromal Tumors (GIST)

Advances in immunohistochemical analysis changed the way gastrointestinal myogenic tumors are classified. Currently, the term "gastrointestinal stromal tumor" (GIST) is used to designate the largest category of primary nonepithelial neoplasms of GI lesions [25–28]. EUS has become a well-established technique for the diagnosis and follow-up of this group of gastrointestinal pathologies. It provides detailed images of GI wall structure and indicates the layer of origin of submucosal tumors. For example, a diagnosis of myogenic tumor can be made confidently when a submucosal tumor is seen to arise from the fourth hypoechoic sonographic layer (muscularis propria) of the adjacent normal GI tract wall. However, the differentiation between benign and malignant myogenic tumors can be endosonographically difficult. Several criteria have been suggested to indicate malignancy including size (> 3 cm), irregular border and the presence of internal cystic spaces [25,29,30]. Surgical resection has classically been recommended for those lesions with suspicious features. Standard endoscopic biopsies typically do not help in the diagnosis of submucosal lesions.

When compared to lymph nodes and pancreatic masses, EUS-FNA is reported to be less useful for the histopathologic diagnosis of submucosal tumors [31,32]. However, if an adequate sample can be obtained, it is possible to count mitotic figures, the most significant indicator of biologic behavior [33,34]. By using the EUS-FNA technique, adequate specimens are usually obtained for histology and differential immunophenotyping of GISTs. With advances in immunohistochemistry, several investigators

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have reported that most myogenic tumors express CD34 and a growth factor receptor with tyrosine kinase activity termed KIT. This receptor, the product of the proto-oncogene c-*kit* (located on chromosome 4q11-q12), can be detected by immunohistochemical staining for CD117. All GISTs are immunohistochemically positive for KIT (CD117) [27,28,25]. Although many other tumor types could be CD117 positive, most of them do not occur in the gastrointestinal tract. Positive CD117 staining (and in most cases positive CD34 staining) in the appropriate clinical setting, with compatible gross and microscopic findings, are the defining features of these tumors [36]. Moreover, tumor immunophenotype is reported to strongly reflect histologic behavior of GISTs [37,38]. For example, Newman et al [39] reported that tumors with a neural phenotype have the best prognosis. However, the results of these studies were based on analysis of surgically resected lesions.

As with pancreatic lesions, the importance of combining EUS and pathology has been emphasized. In a study by Nobuhiro et al [40], preoperative EUS alone and in combination with histopathology/immunohistochemistry was evaluated. The overall accuracy for the diagnosis of malignant GIST was 78% by EUS imaging alone and 91% by histopathologic evaluation (H&E staining) of specimens obtained by EUS-FNA. Immunohistochemical expressions of several markers including c-kit, CD34, muscle actin, S-100 were evaluated on EUS-FNA and post surgical resection specimens. For the diagnosis of malignant GIST, the accuracy, sensitivity, and specificity of EUS-FNA with the addition of Ki-67 immunohistochemical staining were 100%. Further studies are needed to confirm this finding and reevaluate the rest of the markers.

Conclusion

EUS plays an important role in the diagnosis of GI cancers. It has helped shift the current practice from simple staging to early detection and treatment. The recent scientific leaps in the recognition of genetic markers and phenotypic molecular expressions have largely contributed to this shift. The best approach for early cancer detection seems to rely on the coupling of EUS-FNA and available gene markers and immunoassays.

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