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A comparison of surgical and endoscopic sample collection for pancreatic cyst fluid biomarker identification

Katie Partyka¹, Mitchell McDonald¹, Kevin A. Maupin¹, Randall Brand², Richard Kwon³, Diane M. Simeone³, Peter Allen⁴, and Brian B. Haab^{1,*}

¹Van Andel Research Institute, Grand Rapids, MI

²University of Pittsburgh School of Medicine, Pittsburgh, PA

³University of Michigan Medical School, Ann Arbor, MI

⁴Memorial Sloan Kettering Cancer Center, New York, NY

Abstract

Significant efforts are underway to develop new biomarkers from pancreatic cyst fluid. Previous research has made use of cyst fluid collected from surgically-removed cysts, but the clinical implementation of biomarkers would use cyst fluid collected by endoscopic ultrasound-guided, fine-needle aspiration (EUS-FNA). The purpose of this study was to investigate the clinical applicability of cyst fluid research obtained using surgical specimens. Matched pairs of operating-room collected (OR) and EUS-FNA samples from 12 patients were evaluated for the levels of three previously described biomarkers, CA 19-9, CEA, and glycan levels detected by wheat germ agglutinin on MUC5AC (MUC5AC-WGA). CA 19-9 and MUC5AC-WGA correlated well between the sample types, although CEA was more variable between the sample types for certain patients. The variability was not due to the time delay between EUS-FNA and OR collection or differences in total protein concentrations but may be caused by contamination of the cyst fluid with blood proteins. The classification of each patient based on thresholds for each marker was perfectly consistent between sample types for CA 19-9 and MUC5AC-WGA and mostly consistent for CEA. Therefore, results obtained using OR-collected pancreatic cyst fluid samples should reliably transfer to the clinical setting using EUS-FNA samples.

Introduction

Certain types of pancreatic cystic lesions are completely benign and do not pose a risk for progression to invasive cancer, including serous cystadenomas, retention cysts, and pseudocysts, while other types of cystic lesions, such as intraductal papillary mucinous neoplasms (IPMN) and mucinous cystic neoplasms (MCN), are precursors to invasive cancer^{1, 2}. In order to most effectively treat and manage patients with pancreatic cysts, the accurate diagnosis of the type of cyst and the potential for a cyst to progress to invasive cancer is critical^{3–6}. Patients with cysts that are judged to pose a risk of invasive cancer typically are recommended to undergo surgical resection, while patients with less concerning cysts may be preferentially recommended to be enrolled in a surveillance program, without the need for surgical intervention. Both approaches carry a unique set of risks and benefits associated risks. While surgical resection will definitely treat the cyst, the procedure is burdensome to the patient and brings risks of complications and long-term consequences. In addition, the decision to forego surgery could result in the lesion progressing beyond a treatable stage. A surveillance program, with serial analysis of the cyst

*Correspondence to: Brian B. Haab, Van Andel Research Institute, 333 Bostwick NE, Grand Rapids, MI 49503, Brian.haab@vai.org.

with either cross-sectional imaging or endoscopic ultrasound analysis, avoids the need for surgery, but is costly in the long term and has the potential to miss an occult malignancy. The evaluation of pancreatic cysts is currently based on size, location, imaging features, and, if available, analysis of the fluid removed from the cyst by fine-needle aspiration^{3-5, 7, 8}. Unfortunately, these methods are severely limited in the information they provide. Imaging and cyst fluid analysis provides some guidance on the type of cyst but very little on whether the cyst has a high potential for near-term progression to invasive cancer⁸⁻¹⁰. Consequently, many cysts are removed that likely represent no threat to the patient.

The cyst fluid is a rich resource for the discovery of new biomarkers because it is trapped in direct contact with the involved cells, resulting in secreted molecules being retained and concentrated, as opposed to diluted in the circulation. Research groups are studying cyst fluid contents for the development of new biomarkers using approaches including proteomics, glycoproteomics, DNA analysis, microRNA analysis, and others¹⁰⁻¹⁹. For these studies, the cyst fluid is often obtained after surgical removal of the cyst in order to ensure an accurate analysis of the cyst type, as imaging alone is not accurate in confirming cyst type. The definitive diagnosis provided by surgical pathology is necessary for properly designing and interpreting research results. However, in the diagnostic setting, cyst fluid is obtained through imaging-guided biopsy, such as endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA)²⁰. Therefore it is critical to confirm that cyst fluid analysis results derived from samples obtained after surgery apply to samples obtained by fluid aspiration in the diagnostic endoscopic setting. The correspondence between the two collection types in the resulting levels of particular molecular markers is not known, and the eventual clinical performance of biomarkers discovered using surgical specimens is hard to predict without a direct comparison.

The goal of this study was to examine the correlation in specific marker levels between cyst fluid obtained by EUS-FNA and cyst fluid obtained immediately after surgical resection. We assembled a set of matched samples in which both types of samples were available from each patient. The molecular markers we examined were CEA and two additional biomarkers that were previously shown useful for discriminating mucin-producing cysts from non-mucin-producing cysts²¹. We showed previously that the measurement of a particular carbohydrate epitope on the MUC5AC molecule served as a valuable biomarker for pancreatic cysts, and this biomarker, in combination with CA 19-9, distinguished most potentially-malignant cysts from benign cysts. The measurements were made using antibody microarrays, which enable analyses of several different markers using very small amounts of sample^{22, 23}.

Experimental Section

Patients and Cyst Fluid Samples

The study was conducted in strict compliance with the guidelines of the University of Michigan and the Memorial Sloan Kettering Cancer Center Institutional Review Boards. After obtaining signed informed consent, cyst fluid samples were collected at the time of endoscopy or operation at the University of Michigan and the Memorial Sloan Kettering Cancer Center. All patients enrolled in the study had operative treatment of the cystic lesion and the surgical pathology report was used to confirm the diagnosis of cyst type in all patients. A 22 or 25 gauge needle was used for the EUS-FNA and a 19 or 21 gauge needle for FNA from the surgically-removed cysts, depending on the preference of the physician. For the surgical specimens, the fluid was aspirated from an intact, closed cyst. Cyst fluid specimens were immediately placed on ice and were aliquoted and stored at -80°C within an hour of collection. Each sample was thawed no more than three times prior to analysis in order to minimize variability introduced by that process. Each sample was centrifuged at

10,000 × g for 10 minutes to remove remaining debris prior to use. The samples were randomized in their handling and experimental processing.

Biological reagents

Anti-MUC5AC was purchased from AbD Serotec (clone 45M1); CA 19-9 mAb was purchased from US Biological (clone 9L426); and the anti-CEA antibodies were purchased from Abcam (ab4451 as capture antibody and ab15987 as detection antibody). Biotinylated wheat germ agglutinin was purchased from Vector Laboratories (item B-1025). The antibodies were purified by dialysis (Slide-A-lyzer, Pierce Biotechnology, Rockford, IL) to PBS buffer and ultracentrifuged before the concentration of each antibody was adjusted to 500 µg/ml for microarray printing. The integrity and purity of each antibody was confirmed by SDS-PAGE under reducing and non-reducing conditions. Antibody biotinylation was performed using EZ-Link-sulfo-NHS-LC-biotin (Pierce Biotechnology, Rockford, IL). The CEA protein standard was purchased (Fitzgerald Industries, North Acton, MA, #30-AC32).

Microarray fabrication and preparation

Antibody microarrays were prepared as previously described²⁴. A microarrayer (2470 Arrayer, Aushon Biosystems, Billerica, MA) was used to spot subnanoliter droplets of each antibody solution on the surfaces of ultrathin nitrocellulose-coated glass microscope slides (PATH slides, GenTel Biosciences, Madison, WI). Forty-eight identical arrays were printed on each slide, with each array consisting of the antibodies targeting the proteins of interest, as well as control immunoglobulins from several species, printed in triplicate. A wax border was imprinted around each of the arrays to define hydrophobic boundaries (SlideImprinter, The Gel Company, San Francisco, CA). The printed slides were stored at 4°C in a desiccated, vacuum-sealed slide box until use.

Sandwich assays

Assays were performed similar to previously described methods^{24, 25}. Cyst fluid samples were diluted with PBS buffer containing 0.1% Brij's, 0.1% Tween-20 and 50 µg/ml of protease inhibitor. An IgG/IgY cocktail consisting of a final concentration of 400 µg/ml goat, mouse and sheep IgG, 400 µg/ml chicken IgY and 800 µg/ml rabbit IgG (Jackson ImmunoResearch, West Grove, PA) was added to each cyst fluid sample to eliminate non-specific binding to the printed antibodies. Slides were blocked in solution containing PBS-0.5% Tween-20 buffer (PBST0.5) with the addition of 1% BSA. Additionally, samples were spun at 16,000×g for 3 minutes to separate viscous components. Six µl of sample was then applied to each array. Captured antigens were detected with biotinylated detection reagents (either anti-CEA, CA 19-9 monoclonal antibody, or wheat germ agglutinin) at a concentration of 1–10 µg/ml, followed by incubation with 1 µg/ml streptavidin-phycoerythrin (Roche Applied Science, Indianapolis, IN). The slides were scanned for fluorescence emission at 575 nm using a microarray scanner (LS Reloaded, TECAN, Durham, NC). All arrays assaying the same glycoprotein were scanned concurrently at a single laser power and detector gain setting.

Protein concentration assays

The total protein concentration of each cyst fluid sample was determined using the bicinchoninic acid assay (Micro BCA Protein Assay Kit, # 23235, Thermo Scientific) according to the manufacturer protocol. Each sample was diluted 50X into 1X PBS followed by 1:1 dilution with the working reagent. The average value from triplicate measurements was used for each sample.

Data analysis

Image data were quantified using GenePix Pro 5.1 (Axon Instruments, Union City, CA). The net fluorescent signal was calculated by subtracting the median local background surrounding each spot from the median intensity of the corresponding spot. The signal intensities from replicate antibody measurements within the same array were averaged. CEA concentrations were calculated from fluorescence values using a four-parameter polynomial fit to a CEA dilution curve. Data processing and preparation was performed using Microsoft Excel, MultiExperiment Viewer, and Canvas X.

Results

Correlations between the sample types in the levels of three markers

In order to study the relationship in particular molecular markers between cyst fluid samples collected in the operating room (OR) and cyst fluid samples collected by endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA), we assembled pairs of matched samples for which both types of samples were available from each patient (Table 1). Ten pairs were from IPMNs, and two were from MCNs. The use of antibody microarrays allowed the acquisition of several replicate measurements on three different markers using less than three microliters per assay. We previously showed that the detection of specific glycans on specific proteins can yield improved biomarker performance relative to detecting just the core protein level^{25, 26}. Such measurements can be obtained using lectin probing of the glycans on proteins captured by antibody arrays, termed antibody-lectin sandwich arrays^{23, 25}. For the discrimination of mucin-producing cysts from non-mucin-producing cysts, the detection of a glycan epitope on the protein MUC5AC using the lectin wheat germ agglutinin (WGA) provided a valuable marker (designated as MUC5AC-WGA)²¹. MUC5AC previously was shown to be elevated in mucinous cysts, but we found that detection with WGA further enhanced performance. In addition, we found that the detection of the CA 19-9 marker together with MUC5AC-WGA gave better performance than either alone. This combination marker also was significantly better than CEA.

We therefore evaluated the levels of the three markers, MUC5AC-WGA, CA 19-9, and CEA, in the matched cyst fluid samples (Fig. 1A). Each assay was performed in duplicate (for the first two markers) or triplicate (for CEA), and each sample was diluted by the proper amount to bring the given analyte into the linear range of the assay. A comparison of the CA 19-9 values in the EUS-FNA and the OR samples shows a statistically significant correlation ($p < 0.05$). That is, patients with a high CA 19-9 value in their EUS-FNA sample also had a high value in their OR sample. The variability seen between the EUS-FNA and the OR samples is only slightly more than the inherent variability in the assay (Fig. 1B). MUC5AC-WGA likewise showed good general correlation ($p < 0.05$) between the two sample types. However, CEA did not show as good agreement between the sample types. Certain patients had very high levels in the OR sample but not in the EUS-FNA sample (such as patients 3 and 12), and vice versa (patient 4). The replicate CEA assays had good reproducibility (Fig. 1B) (CVs between the triplicate measurements were all less than 0.4), so the differences between the OR and EUS-FNA samples do not reflect variability in the assay. These data suggest that the levels of certain markers are consistent between the two sample types, yet other markers such as CEA have more variability.

Potential sources of variation between the sample types

We further investigated potential sources of variability between the EUS-FNA and the OR samples. An unknown factor was whether variation in total protein concentration could account for the differences between samples in the concentration of a given marker. We found that the protein concentrations generally correlated between the EUS-FNA and the

OR samples (Fig. 2A), although some patients showed a major change between the EUS-FNA and the OR samples, such as patients 3, 4, and 9. No overall statistical difference in concentrations was observed between the OR and the EUS-FNA samples.

Because the concentrations were highly divergent between the patients, with a few having extremely high concentrations (>30 mg/ml) and some very low (Fig. 2A), we asked whether the total protein concentration influenced the level of any of the markers. The samples with extremely high concentrations likely contain high levels of blood that occurred during sample acquisition, so if the levels of a particular marker correlated well with total protein concentration, the marker levels might simply reflect blood levels. A cluster of all the marker measurements and the total protein concentrations is a convenient way to view overall correlations (Fig. 2b). The total protein concentrations did not correlate with the levels of any of the markers, indicating that the marker concentrations were not simply reflective of total protein concentration. (The general agreement between the EUS-FNA and the OR values is indicated by the adjacent clustering of those values for each marker.) However, we observed some indication that the change in total protein concentration from the EUS-FNA sample to the OR sample correlated with the change in CEA levels. For example, patients 3 and 4 had major differences in total protein concentration between the EUS-FNA and the OR samples (Fig. 2A) which is mirrored by the differences in the CEA values for the same patients (Fig. 1). It is possible that CEA from blood is contaminating the cyst fluid, which alters both the total protein concentration and the CEA levels at the same time.

Another factor that might introduce variation between the EUS-FNA samples and the OR samples for CEA is the amount of time that passed between the collection of the two samples. For example, matched samples with a greater amount of time between collections could have greater differences in marker levels. To test this possibility, we plotted the relative difference in signal between the EUS-FNA samples and the OR samples with respect to time differential (ranging from 8 to 250 days) in sample collection (indicated in Table 1) for each matched pair of samples (Fig. 3). No association was observed for CA 19-9 and CEA, and a slight positive association was observed for MUC5AC-WGA ($p = 0.05$), indicating that the time differential between sample collections is not a major factor contributing to changes in the levels of the markers.

Comparison between the sample types in patient classification

A critical question is whether the eventual conclusions for biomarker studies are different between those obtained using OR samples and those obtained using EUS-FNA samples. We previously investigated a two-marker panel in which an elevation in either CA 19-9 or MUC5AC-WGA indicated a mucin-producing cyst, as opposed to a non-mucin-producing cyst²¹. The present study did not include control cysts (non-mucin-producing) for comparison, so we set an arbitrary threshold at the top of the linear range for each marker to indicate which samples were positive and which were negative. The identical patients were positive in either marker when using just the EUS-FNA samples or using just the OR samples (Fig. 4A), indicating that the result obtained using the OR samples should accurately transfer to the EUS-FNA samples. For the CEA values, we applied a threshold of 200 ng/ml, which was previously used to distinguish potentially malignant from benign cysts¹⁰. Four patients were below the threshold using the EUS-FNA samples, and using the OR samples, two of the four were still low and two had become high (Fig. 4B). The two patients that switched, patients 8 and 12, did not have major changes in protein concentration, so it appears that the CEA level accumulated in the intervening time between the EUS-FNA collection and the OR collection. Other patients that had major changes in CEA concentration, such as patients 3 and 4, did not have changes relative to the threshold of 200 ng/ml. Therefore, while some variability exists in the CEA levels between EUS-FNA

and OR samples, greater consistency is observed at the clinically-relevant threshold of 200 ng/ml. The more precise agreement for the other two markers suggests that such consistency is common and supports the conclusion that results obtained using OR samples should be reliably applied to EUS-FNA samples.

Discussion

The development of biomarkers requires samples with accurate annotation, so that precise determinations can be made about the associations between particular markers and disease. For cyst fluid samples, a definitive diagnosis is currently only available if the cyst has been removed and examined by surgical pathology. For that reason, a practical means of assembling sample sets that are informative for research has been to collect cyst fluid after surgical resection of the cyst. While such samples have the benefit of having an associated definitive diagnosis, their correspondence to samples collected by endoscopic fine needle aspiration, which is the mode used in the diagnostic setting, was unknown. Here we examined that correspondence by comparing several known biomarker levels and the total protein concentrations between matched EUS-FNA and OR samples from 12 patients. Our findings have important practical implications both for biomarker research and for the clinical use of biopsy-collected pancreatic cyst fluid.

Two of the three markers, CA 19-9 and MUC5AC-WGA, agreed well between the sample types, indicating that for certain markers, results obtained using OR samples should translate directly to EUS-obtained cyst fluid aspiration samples. The CEA marker showed major differences between the sample types for four of the 12 patients. These differences were not due to variability in the assay or variation in the time differential between EUS-FNA and OR sample collection, but they were related to changes in total protein concentration for two of the patients. For those patients, a major change in total protein concentration from the EUS-FNA sample to the EUS sample mirrored a corresponding change in CEA concentration. The most likely explanation for this relationship is that CEA was introduced into the cyst fluid from contaminating blood, which can contain elevated levels of CEA²⁷. Such a source of CEA would cause changes in total protein concentration to correlate with changes in CEA concentration. This possibility could be further tested by examining the CEA concentrations in patient-matched samples from cyst fluid and blood serum. Patient-matched blood serum samples were not available for analysis for the patients included in this study, but this analysis will be incorporated in future studies. Another source of change in the CEA levels could be rapid and unpredictable turnover in the protein levels due to proteases that are present in cyst fluid²⁸. We sought to minimize such an effect through the treatment of our samples with a high level of broad-specificity protease inhibitors, but the possibility remains that unforeseen modes of CEA clearance are active. Since CEA is a widely-used clinical biomarker for pancreatic cyst fluid, the implications of this result on the overall performance for diagnosing pancreatic cysts should be further investigated.

Despite the variability observed in CEA for certain patients, the agreement between sample types in which patients were classified as above or below the clinical threshold of 200 ng/ml was high, with all but two patients classified the same. In the final analysis, the simple elevation or lack of elevation of a marker is the important consideration, and variability among the high samples is less important. By that standard, CEA results based on OR samples should reliably transfer to EUS-FNA samples. Furthermore, the more precise agreement in the MUC5AC-WGA and the CA 19-9 markers suggests that good correspondence between the sample types is common and supports the clinical applicability of research results obtained using OR samples.

This study also enabled a look at variation in cyst fluid total protein concentration and the affects of that variation on biomarker measurements. Cyst fluid is different from plasma or serum in that the composition and total protein concentration is much more variable between patients. Serum total protein concentrations are regulated by the high levels of albumin and other major blood proteins, but cyst fluid arises from an abnormal cavity that has no such biological regulation. Since the variation in total protein concentration was equally present in the EUS-FNA and the OR samples, it was not the result of the mode of sample collection. Rather it likely reflects inherent differences between the cysts in the amount of blood protein contamination. This possibility could be tested by examining the correlation between total protein concentration and levels of major serum proteins such as albumin in the cyst fluid. The fact that total protein concentration does not generally correlate with the biomarker levels is valuable for the practical implementation of cyst fluid biomarkers, since serum contamination does not greatly affect individual measurements.

In a similar way, the lack of correlation of the marker measurements with time differential between the EUS-FNA and the OR samples is useful information for biomarker research using OR samples. This result indicates that cyst fluid protein concentrations and individual biomarker concentrations are relatively stable over the 8 day to 250 range examined here, meaning that the timing of the fluid aspiration will not greatly affect results.

While the major differences between samples in total protein concentration did not greatly affect the individual markers measured here, these differences may have consequences for proteomics-based discovery research, such as those previously used to analyze cyst fluid^{29, 30}. The presence of high abundance proteins in a sample can reduce the number of proteins that are detectable using mass spectrometry-based protein discovery methods. A common approach to enable increased identifications from blood serum is to deplete the sample of major serum proteins. Since the abundance of such proteins is less predictable in cyst fluid, proteomics research could include an initial evaluation of each cyst fluid sample to determine whether the depletion of high abundance proteins would be beneficial.

This work should help to further establish the foundation for using cyst fluid both in research setting and in a diagnostic setting. Biomarker research performed using OR-collected samples should translate directly to biopsy samples, based on the good correspondence between the sample types observed here. Since the marker levels appear to be generally stable over time, variation in the timing of a biopsy will not affect results. Furthermore, the major variation in total protein concentration observed between cyst fluid samples does not confound individual marker measurements, although contamination of the cyst fluid by blood could alter values provided the biomarker is also found in detectable quantities in the blood. These findings confirm the suitability of protein markers in cyst fluid for clinical diagnostics. However, given the major variation between samples in total protein concentration and levels of blood contamination, the evaluation of new cyst fluid markers should include analyses of the effects of blood contamination on marker measurements and clinical performance.

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Figure 1.

Correlation between OR samples and EUS-FNA samples in the values obtained for three markers. A) For each patient, the value of the indicated marker obtained from the OR sample (y-axis) is plotted with respect to the value obtained from the EUS-FNA sample (x-axis). The best fit linear trendline is indicated. The dashed lines represent the upper limit of the assay linear range for CA 19-9 and MUC5AC-WGA and the clinical threshold of 200 ng/ml for CEA. B) The reproducibility of each assay is indicated by plotting values from one set of data with respect to values from a replicate set. Two representative replicates out of three are shown. Trendlines are presented separately for the OR samples and the EUS-FNA samples, showing similar reproducibilities. The replicate CEA measurements were

taken on the same day, and the replicate measurements for the other markers were on different days with different batches of microarrays. RFU, relative fluorescence units.

Figure 2.

Variation in total protein concentration and effect on individual measurements. A) Correlation in total protein concentrations between the OR samples and the matched EUS-FNA samples. Each point represents data from one patient, with the y-axis value indicating the OR sample measurement and the x-axis value indicating the EUS-FNA sample measurement. The left panel shows the entire data set, and the right panel shows a detailed view of the low concentration samples. B) Correlations between all markers and total protein concentration. The measurements from the three markers in the EUS-FNA and OR samples (indicated by the row labels) for all patients (indicated by the column labels) were clustered. The value of each measurement is indicated by the color bar. The adjacent rows for each marker show the overall correlation between the EUS-FNA and the OR samples. The total protein concentration has a distinct pattern from any of the markers.

Figure 3.

The effect of time lag in sample collection on variation in marker levels and total protein concentrations. For each marker and total protein concentration, the relative difference between the EUS-FNA measurements and the OR measurements are plotted with respect to time differential in sample collection (determined as the number of days between the dates of EUS-FNA sample collection and OR sample collection). The best linear fit is included in each graph.

Figure 4.

Classification of the samples based on the marker levels. Thresholds were applied to each of the markers to classify samples as elevated or not elevated in each marker. Using CA 19-9 and MUC5AC-WGA, the patient classification was identical between the EUS-FNA samples and the OR samples. The patient classification using CEA at a threshold of 200 ng/ml showed that only patients 3 and 11 changed classification from the EUS-FNA sample to the OR sample.

Table 1

Patient and sample characteristics.

Patient	Diagnosis	Time between samples (days)*
1	Neuroendocrine cyst	14
2	IPMN with carcinoma in situ	33
3	IPMN, moderate grade	95
4	IPMN, moderate grade	42
5	IPMN, moderate grade	71
6	IPMN, moderate grade	122
7	IPMN, low grade	43
8	IPMN, low grade	34
9	MCN. Low grade	250
10	IPMN	8
11	IPMN	39
12	IPMN	15

* Days between the OR-EUS sample collection and the OR sample collection.