Biomarkers in Diagnosis of Pancreatic Carcinoma in Fine-Needle Aspirates

A Translational Research Application

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Key Words: Gastrointestinal; Cytopathology; Cytology; Fine-needle aspiration; Biological markers; Pancreatic carcinoma; Endoscopic ultrasound; Translational research

DOI: 10.1309/CEV30BE088CBDQD9

Abstract

*This study was undertaken to determine whether recently identified proteins could be translated to clinical practice as markers to distinguish pancreatic adenocarcinoma from chronic pancreatitis on fine-needle aspirate (FNA) samples. Resected pancreatic tissue sections (n = 40) and FNA samples (n = 65) were stained for clusterin-β, MUC4, survivin, and mesothelin. For each biomarker, the staining patterns in adenocarcinoma and in reactive ductal epithelium were evaluated and compared. Clusterin-β stained reactive ductal epithelium significantly more frequently than pancreatic adenocarcinoma (*P < *.001). In comparison, MUC4 and mesothelin were expressed more frequently in pancreatic adenocarcinoma on tissue sections. Positive staining for MUC4 (91% vs 0%;* P < *.001) and mesothelin (62% vs 0%;* P = *.01) and absence of staining for clusterin-β (90% vs 7%;* P < *.001) were noted significantly more frequently in adenocarcinoma cells than in reactive cells in FNA samples. Clusterin-β and MUC4 can help distinguish reactive ductal epithelial cells from the cells of pancreatic adenocarcinoma in FNA samples.*

Pancreatic carcinoma is the fourth leading cause of cancer-associated deaths in the United States. The majority of these tumors occur at advanced stages, and, therefore, resection may not be an option. Our study and studies by others show that resection of small (early) pancreatic tumors (<3.0 cm) and tumors of low histologic or cytologic grade and stage are correlated with improved survival.[1,2] It is, therefore, important to identify these tumors in their early stages.

In a patient clinically suspected to have a pancreatic tumor, imaging by conventional computed tomography scanning has become one of the standard modalities to indicate the size and extent of the tumor. Recently, it has been documented that endoscopic ultrasound (EUS) is a more sensitive modality than imaging by conventional computed tomography scan for detecting small pancreatic tumors and for detecting invasion of vessels, which may help to assess resectability of pancreatic carcinomas.[3-5] Imaging modalities alone, however, can neither differentiate benign from malignant lesions nor determine the type of neoplasm. The National Comprehensive Cancer Network consensus group, formed of experts treating pancreatic cancer, also has recognized that tissue diagnosis should be obtained before instituting definitive therapy.[6] In cases in which imaging studies suggest an unresectable pancreatic tumor, a diagnosis of adenocarcinoma on cytologic assessment of fine-needle aspirate (FNA) becomes important before starting protocol-associated therapy.[6]

A preoperative tissue diagnosis can be obtained by using image-guided FNA and needle biopsies. In recent years, EUS-guided FNA has been used increasingly to obtain preoperative diagnosis and for staging pancreatic carcinoma.[4,5] This application of EUS-FNA is becoming common since it has emerged as a very sensitive and specific modality in the diagnosis of pancreatic

adenocarcinoma.[7-9] Furthermore, we also have demonstrated that the sensitivity and specificity of detecting pancreatic carcinoma using EUS-FNA does not vary whether the lesion is small or large.[7] Although many studies suggest improved specificity, experts agree that interpreting FNA samples from the pancreas is inherently difficult and that interpretation is even more challenging when samples are scant and bloody, as often noted on EUS-FNA.[10] In our experience and that of others, nondiagnostic samples (inadequate or equivocal diagnoses) from pancreas and other organs when using EUS-FNA range from 11% to 30%.[7,8,11,12] In such a scenario, a marker that can serve as an adjunct in separating the cells of pancreatic adenocarcinoma from the cells of reactive ductal epithelium would be very useful.

Recently, the use of high-throughput technologies on multiple pancreatic cell lines has identified potential genes and proteins that could have roles in pancreatic carcinogenesis. It would be important to determine the clinical usefulness of such differentially regulated proteins, especially if they could be used as markers to separate the cells of reactive ductal epithelium from the cells of pancreatic adenocarcinoma. FNA procedures guided by EUS imaging are increasingly being performed throughout major centers to obtain preoperative diagnostic and staging information; thus, performing biomarkers on the limited samples obtained using EUS-FNA could become extremely important for multidisciplinary patient management teams if they were useful in aiding the diagnosis of pancreatic adenocarcinoma.

Clusterin/apolipoprotein J is a secreted heterodimeric glycoprotein of 70 to 80 kd.[13] Clusterin (apolipoprotein J) also serves as a heat shock protein and has chameleon-like activity that influences many basic cell functions, including cell remodeling, differentiation, apoptosis, and cell proliferation.[14-16] As a result, it has been proposed as a candidate for the development of antisense therapies for certain tumors.[17] Clusterin has been reported to be overexpressed in anaplastic lymphoma[18] and various carcinomas.[19,20] In experimental studies, overexpression of clusterin was associated with regeneration and development of pancreas and pancreatitis.[21,22]

MUC4 is a transmembrane apomucin that has been reported to be overexpressed in pancreatic cancer cells.[23] The messenger RNA for MUC4 also has been identified as overexpressed in more than 75% of pancreatic adenocarcinomas but not in chronic pancreatitis.[23] Expression of MUC4 also has been reported to be a prognostic indicator for pancreatic adenocarcinoma.[24] Decreased expression of MUC4 induced in a pancreatic cell line xenograft via plasmid-transfected anti-MUC4 led to decreased expression of MUC4, reduced clonogenicity, decreased cell proliferation, and reduced tumor volume.[25] These studies suggest that MUC4 expression has an important role in the development and progression of pancreatic adenocarcinoma. Its usefulness as a diagnostic marker in limited samples remains unexplored.

Survivin, described in 1997,[26] is an inhibitor of apoptosis. Overexpression of survivin in human pancreatic tissues is postulated to be an early molecular event in pancreatic carcinogenesis,[27] and its expression has been correlated with prognosis[28]; however, to our knowledge, there has been no study to characterize its usefulness as a marker to aid in preoperative diagnosis using FNA samples.

Mesothelin is a differentiation antigen expressed on the cell membrane of normal mesothelial cells. Recent gene expression data and serial analysis of gene expression also demonstrated that mesothelin could be expressed not only by mesotheliomas and ovarian epithelium but also by pancreatic cancer cells.[29] Tissue expression studies also have demonstrated that mesothelin could be expressed in 85% to 100% of pancreatic cancer cells.[29,30]

To our knowledge, however, except for mesothelin, there is not a single study in the literature that shows that the aforementioned candidate markers could be translated to diagnostic markers for limited cytology samples. The present study was undertaken to determine whether the recently identified proteins could be translated to clinical practice as diagnostic markers to distinguish pancreatic adenocarcinoma from chronic pancreatitis on FNA samples.

Materials and Methods

Training Set (Resected Pancreatic Tissues)

Tissue sections from 40 cases of resected pancreas were retrieved from the files of University of Alabama at Birmingham. H&E-stained tissue sections were reviewed further for adjacent chronic pancreatitis, pancreatic intraepithelial neoplasia (PanIN) and pancreatic adenocarcinoma. PanIN was characterized further into PanIN 1, PanIN 2, and PanIN 3 based on criteria defined earlier by a group of experts.[31] For the purposes of the study, we categorized PanIN 1 and PanIN 2 in one group and PanIN 3 in another group.

Test Set (Cytology Samples)

We analyzed 304 FNA samples from pancreas and provided on-site diagnosis and processed samples for further evaluation as described previously.[5] From these cases, we selected only cases aspirated from the head of the pancreas and that had adequate cellularity in the cell blocks. By using these 2 criteria, we selected 65 cases of pancreatic FNA with a diagnosis of chronic pancreatitis (n = 18), atypical or "suspicious" for carcinoma (n = 12), and pancreatic adenocarcinoma (n = 35). All cases of malignancy were confirmed based on progression of disease on imaging studies and/or tissue confirmation (cytology from metastatic site or pancreas resection).

DOI: 10.1309/CEV30BE088CBDQD9

Similarly, lesions were considered benign if there was a lack of progression or resolution on imaging studies in conjunction with continued well-being of the patient for at least 6 months. In addition, when available, a diagnosis of benign or reactive process made on a resected sample or a tissue biopsy sample from the target area also served to confirm the benign nature of the lesions.

Immunohistochemical Stains

Consecutive tissue sections were then stained for the β chain of clusterin, survivin, mesothelin, and MUC4 **Table 1**. Known positive and negative control slides were used to determine the acceptability of the staining reaction. For all stains, cytoplasmic immunoreactivity was considered a positive reaction. The intensity (range, 0 to 4+) of staining and the percentage of positive cells were recorded for each stain in areas with adjacent chronic pancreatitis, PanIN, and pancreatic adenocarcinoma. In addition, nuclear reactivity with or without cytoplasmic staining was considered positive for survivin expression. We used cutoff values for MUC4, mesothelin, and survivin based on our own observations and those reported in previous studies.[24,27,29,30,32] Accordingly, we considered MUC4, mesothelin, and survivin as a positive reaction when more than 5% of cells with a staining intensity greater than 2 were noted in the cytoplasm. For clusterin expression, we considered reactivity in more than 10% of cells as positive. All stains were reviewed independently by 2 pathologists (N.J. and D.J.).

Statistical Analysis

A χ^2 test and a Fisher exact test were performed to determine statistical differences in expression between chronic pancreatitis and pancreatic adenocarcinoma for tissue sections and cytology samples, with the α value set at .05.

Results

Training Set

The mean age of the patients with resected pancreatic carcinoma was 65 years (range, 47-87 years). Tumor sizes ranged from 1.4 to 7.9 cm. The tumors were located in the head of the pancreas. Tumors were categorized histologically as moderately differentiated with foci of poor tumor differentiation constituting 2% to 20% of the entire tumor area. The resected tissues also demonstrated foci of PanIN 1, 2, and 3 and admixed chronic pancreatitis. For staining purposes, we considered PanIN 1 and 2 as 1 group.

There were significant differences in the expression patterns of clusterin-β, survivin, MUC4, and mesothelin. We noted a significant ($P < .001$) difference in the number of cases that expressed clusterin-β in chronic pancreatitis (100%) in comparison with pancreatic adenocarcinoma cases (1%) **Figure 1A** and **Image 1A**. In contrast, immunophenotypic expression of MUC4 (89% vs 1%; $P < .001$) and mesothelin (80% vs 10%; $P = .01$) was expressed significantly more commonly in pancreatic adenocarcinoma than in reactive ductal epithelium **Figure 1B**, **Figure 1C**, **Image 1B**, **Image 1C**, and **Image 1D**. Similarly, survivin expression was noted more frequently in pancreatic adenocarcinoma (70%) than in reactive ductal epithelium (34%), but this difference did not reach statistical significance ($P = .06$) **Figure 1D**, **Image 1E**, and **Image 1F**. As noted in Figure 1A, a decreasing percentage of cases with PanIN and pancreatic carcinoma expressed clusterin-β. In contrast, a progressively increased frequency of samples demonstrated immunoreactivity for MUC4, mesothelin, and survivin in PanIN lesions and pancreatic carcinoma.

Test Set

We obtained 65 FNA samples that were from 38 men and 27 women with ages ranging from 52 to 68 years. Of 12 cases with an indeterminate diagnosis, 8 were reported as atypical and 4 as suspicious for carcinoma. All 4 cases with a diagnosis of suspicious for carcinoma later were confirmed as malignant. Of the 8 atypical cases, 2 were confirmed as negative for carcinoma and demonstrated extensive chronic pancreatitis; 6 subsequently were confirmed as pancreatic adenocarcinoma.

As with the pattern from the training set, negative staining for clusterin-β (3/45 [7%]) **Image 2A** and positive staining for MUC4 (41/45 [91%]) **Image 2B** and mesothelin (28/45 [62%]) **Image 2C** were noted for carcinoma cases. This staining pattern demonstrated significant differences in expression from cases without carcinoma **Table 2**.

Table 1
Immunohistochemical Staining

Antibody	Clone	Pretreatment	Dilution	Company or Supplier
Clusterin-β	B-5	HIER	1:200	Santa Cruz Biotechnology, Santa Cruz, CA
Survivin		HIER	1:400	Lab Vision, Fremont, CA
Mesothelin	5B2 (MESO1)	HIER	1:20	Lab Vision
MUC4	8G7	HIER	1:3,000	S.K.B.

HIER, heat-induced epitope retrieval.

DOI: 10.1309/CEV30BE088CBDQD9

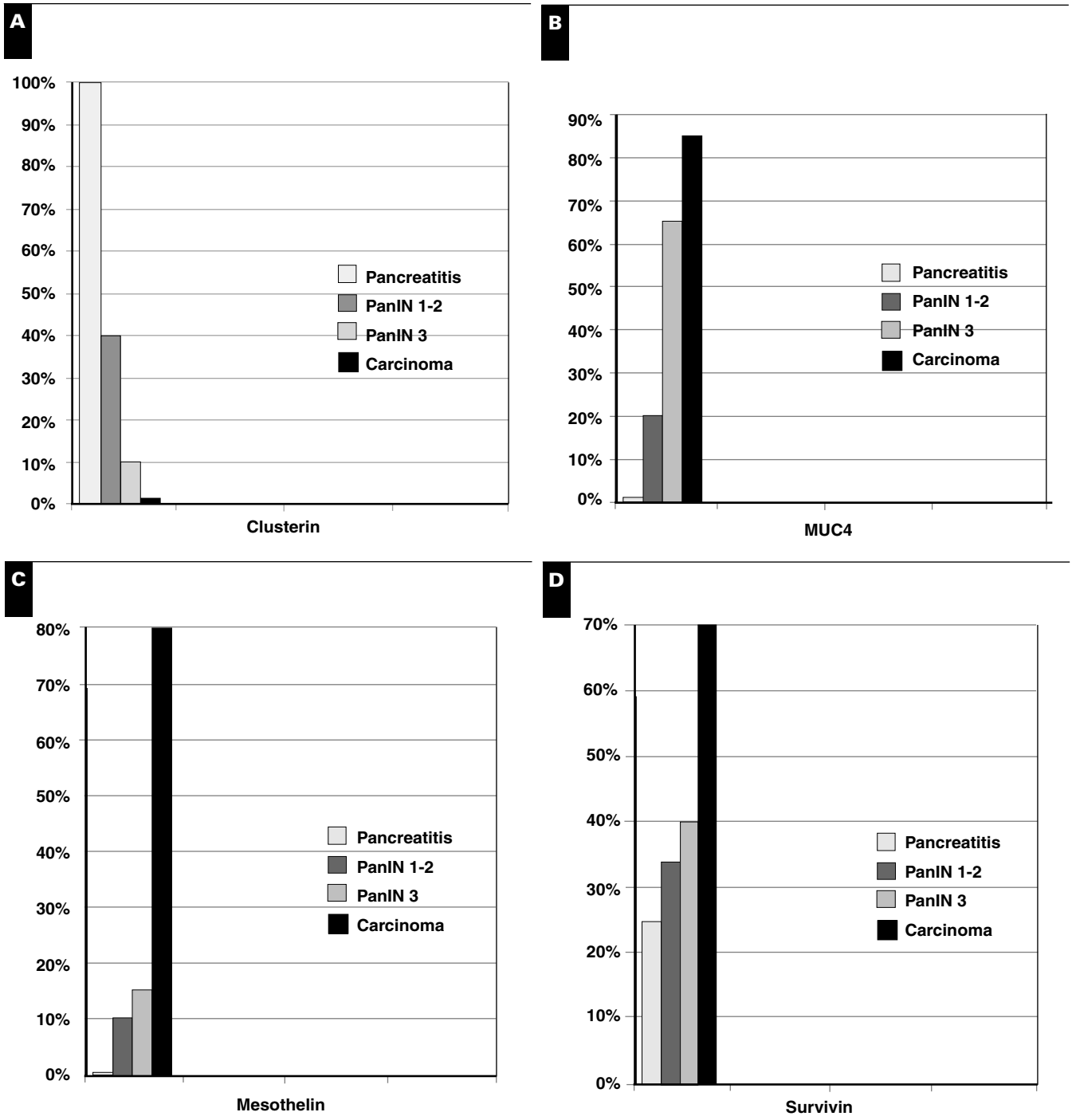

Figure 1 The percentage of cases demonstrating expression of clusterin-β (**A**), MUC4 (**B**), mesothelin (**C**), and survivin (**D**) in resected pancreatic tissues. In chronic pancreatitis vs pancreatic adenocarcinoma, there were significant differences between the numbers of cases expressing clusterin-β ($P = .001$), MUC4 ($P = .001$), and mesothelin ($P = .01$); the difference for survivin expression was not significant ($P = .06$). PanIN, pancreatic intraepithelial neoplasia.

MUC4 expression was absent in 2 of 35 carcinomas and 2 of 12 indeterminate cases that subsequently were confirmed as malignant. Similarly, clusterin expression always was positive in all cases that were considered reactive or atypical and subsequently confirmed as negative for carcinoma. Lower frequency of expression was noted with mesothelin in pancreatic adenocarcinoma cases compared with MUC4; however, when expressed, it always was associated with pancreatic adenocarcinoma.

Operating characteristics for each stain were calculated assuming that negative clusterin-β expression and positive

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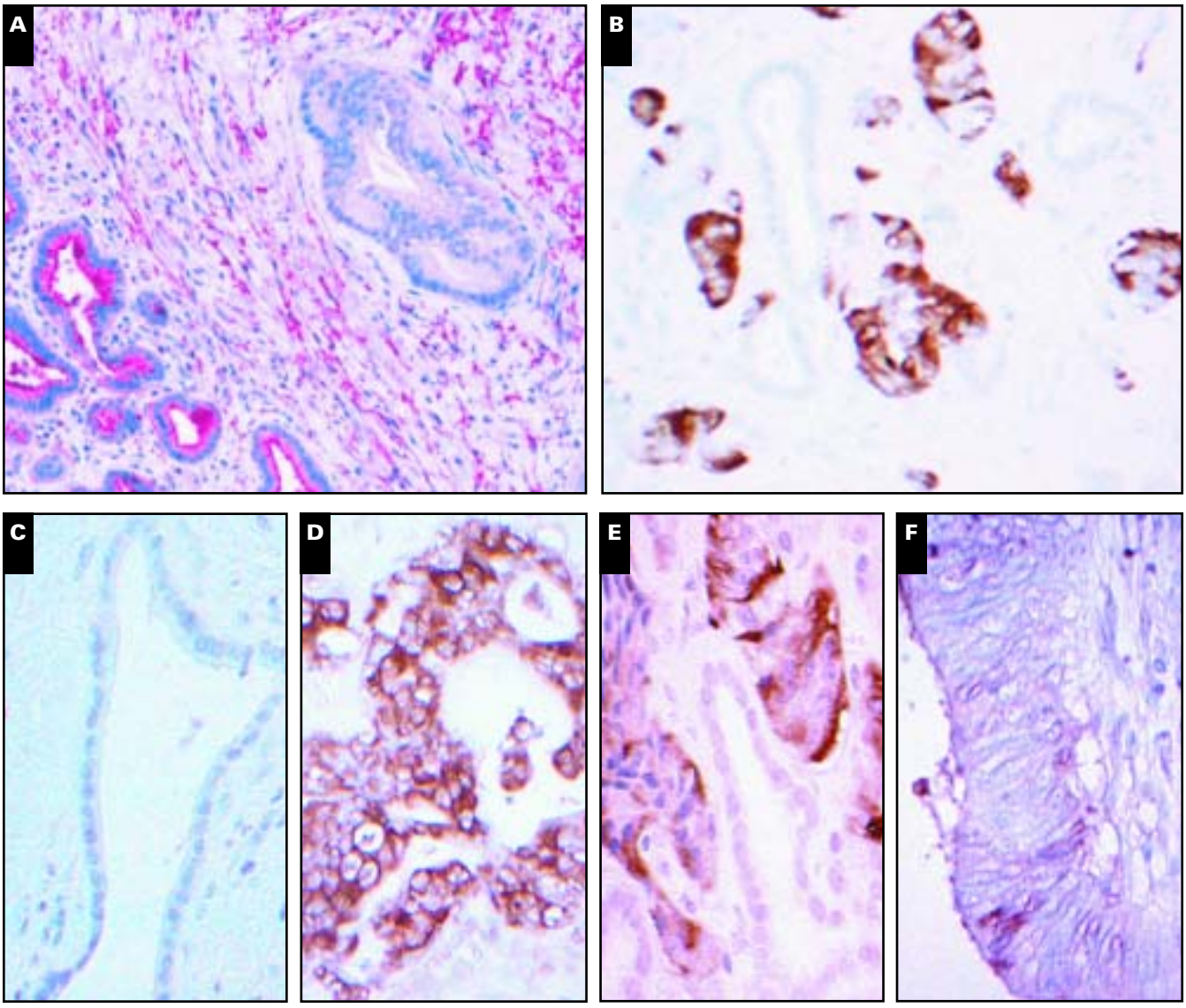

Image 1 **A**, Clusterin-β (red) expression is noted in chronic pancreatitis but not in well-formed glands of pancreatic adenocarcinoma (×20). **B**, MUC4 expression is noted in carcinoma cells but not in the reactive ductal epithelium of pancreas (×20). **C**, Reactive ductal epithelium shows absence of mesothelin immunoreactivity in resected tissues (×40). **D**, Pancreatic adenocarcinoma cells show positive immunoreactivity for mesothelin expression (×40). **E**, Reactive ductal epithelium shows absence of survivin immunoreactivity in resected tissues. Adjacent islet cells demonstrate immunoreactivity for survivin (×40). **F**, Pancreatic adenocarcinoma cells show predominantly cytoplasmic immunoreactivity for survivin expression (×40).

Table 2
Immunophenotypic Expression of Various Markers in Fine-Needle Aspiration Samples in Patients With and Without Adenocarcinoma*

Stain	Reactive (n = 20)	Adenocarcinoma (n = 45)	Significance
MUC4	0 (0)	41 (91)	.001
Clusterin	18 (90)	3 (7)	.001
Survivin	12 (60)	27 (60)	Not significant
Mesothelin	0 (0)	28 (62)	.001

* Data are given as number (percentage).

staining for MUC4 and mesothelin are associated with pancreatic carcinoma. These assumptions demonstrated that expression of MUC4 and mesothelin was very specific for carcinoma, whereas absence of clusterin-β expression was more sensitive and reasonably specific for detecting pancreatic carcinoma **Table 3**. Survivin expression **Image 2D** did not emerge as a marker that could be used reliably to separate reactive ductal cells from pancreatic adenocarcinoma in cytology samples.

DOI: 10.1309/CEV30BE088CBDQD9

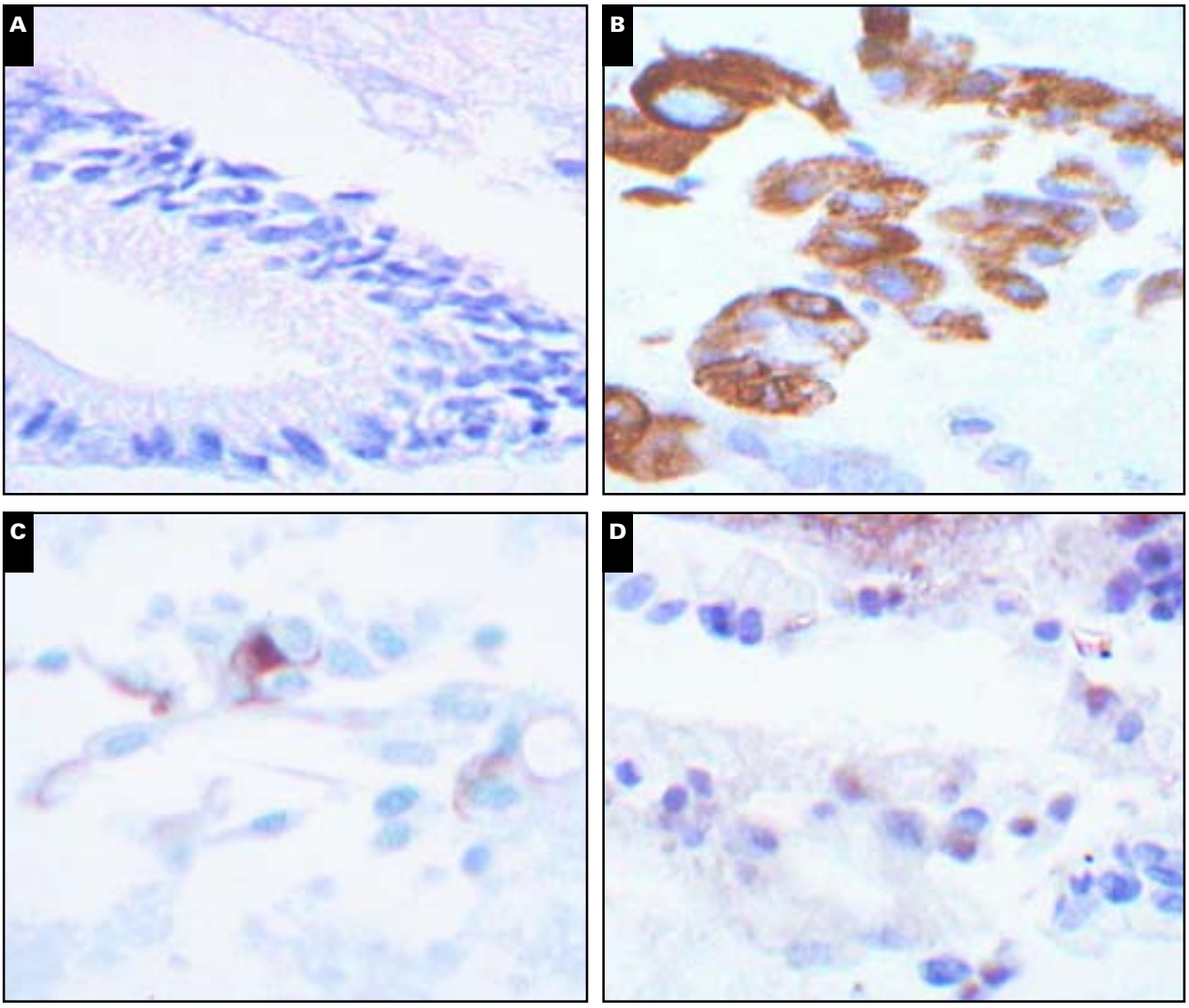

Image 2 **A**, Cell block prepared from a case with well-differentiated pancreatic adenocarcinoma cells that show a lack of clusterin-β expression (×40). **B**, MUC4 expression is noted in a cell-block preparation from a sample with adenocarcinoma (×40). **C**, Mesothelin expression is noted in a few carcinoma cells in a cell-block preparation (×40). **D**, Survivin expression is noted in a few carcinoma cells in a cell-block preparation (×40).

Discussion

Although there are many markers identified based on high-throughput technologies that could separate pancreatic adenocarcinoma from chronic pancreatitis, few have been validated for use in clinical practice. Increasing numbers of biomarkers using the immunophenotypic approach on resected tissues also has been studied in an attempt to demonstrate the pathogenetic basis or prognosis of pancreatic carcinoma. The importance of these markers in diagnostic application, however, remains largely undetermined. A recent review by Manne et al[33] suggests that although high-throughput technologies serve to identify candidate markers, rigorous standards may be needed to help validate the candidate markers. In this changing trend[34] of clinical practice, preoperative diagnosis and staging are being performed

Table 3
Operating Characteristics for Candidate Markers in the Detection of Pancreatic Adenocarcinoma

	Sensitivity (%)	Specificity (%)
MUC4	91	100
Clusterin	93	90
Survivin	60	40
Mesothelin	62	100

DOI: 10.1309/CEV30BE088CBDQD9

increasingly based on a combination of imaging and FNA studies.[5]

Similar to our findings with the training set, Swartz et al[32] demonstrated a progressive increase in immunophenotypic expression of MUC4 in low-grade PanINs and pancreatic adenocarcinoma (sample size, 25 cases). It also has been demonstrated that MUC4 is expressed in almost all cloned pancreatic carcinoma cells and in 12 of 16 human pancreatic carcinoma tissue samples.[23] Our study shows that MUC4 expression could become a useful ancillary study for distinguishing chronic pancreatitis from pancreatic adenocarcinoma, especially when the tissue sample is limited, as is the case with FNA samples.

Our results also demonstrate that absence of immunophenotypic expression of the β chain of clusterin can distinguish chronic pancreatitis from pancreatic adenocarcinoma on limited FNA samples. Recently, Grutzmann et al[35] performed a meta-analysis of 4 published studies using high-throughput technology that demonstrated that although variable expression was noted among the 4 studies, clusterin was expressed differentially in pancreatic adenocarcinoma.[35] Our results are at variance with the study by Xie et al,[36] who reported that immunophenotypic expression of clusterin could be identified in pancreatic adenocarcinoma and that its progressive downregulation was associated with rapid tumor progression. These differences may be a reflection of differences in the staining protocols used to determine immunophenotypic expression. We identified earlier that a lower concentration of clusterin (1:1,000) expression preferentially highlighted glucagon-secreting islet cells but not insulin-secreting cells.[37] The present study documents that at a higher concentration of the β chain of clusterin will differentially highlight reactive ductal epithelial cells but not pancreatic carcinoma cells in resected tissues. A larger study may, however, further validate these findings.

Our study shows that when present, mesothelin is expressed in 62% of cases of pancreatic adenocarcinoma, and its expression can help separate reactive ductal epithelium from pancreatic adenocarcinoma. The present series, using a more stringent method for analysis, confirmed the findings of McCarthy et al,[38] the only previous report, who showed that mesothelin expression can separate pancreatic adenocarcinoma and reactive ductal epithelium on image-guided FNA samples, specifically in 13 (68%) of 19 cases. We confirm the results of this only report in the literature and also show that 28 (62%) of 45 cases of carcinoma expressed mesothelin. Results of both of these studies show that mesothelin expression is observed in fewer cases than reported on resected tissue sections.[29,30] This discrepancy between resected tissues and FNA samples reflects the inherent heterogeneous expression of mesothelin in tissue sections.

We could not confirm that survivin expression can consistently separate chronic pancreatitis from pancreatic adenocarcinoma. However, we showed a trend in that survivin is expressed more frequently in pancreatic carcinomas.

This study demonstrates that candidate markers identified based on rigorous experiments performed on cloned cells and microarray-based technologies can be used as diagnostic markers to separate pancreatic adenocarcinoma from reactive ductal epithelial cells in a diagnostic cytopathology laboratory. Their routine use in clinical practice could become a valuable asset when morphologic features suggest the need for a supportive immunophenotype to confirm the diagnosis of pancreatic carcinoma.

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Supported in part by grant P20CA101955 from the National Institutes of Health–National Cancer Institute–sponsored pancreatic SPORE program (Bethesda, MD).

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DOI: 10.1309/CEV30BE088CBDQD9

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DOI: 10.1309/CEV30BE088CBDQD9