

Staining for p53 and Ki-67 increases the sensitivity of EUS-FNA to detect pancreatic malignancy

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Abstract

AIM: To investigate whether tumor marker staining can improve the sensitivity of endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) to diagnose pancreatic malignancy.

METHODS: Patients who underwent EUS-FNA were retrospectively identified. Each EUS-FNA specimen was evaluated by routine cytology and stained for tumor markers p53, Ki-67, carcinoembryonic antigen (CEA) and CA19-9. Sensitivity, specificity, positive and negative predictive values (PPV and NPV), and positive and negative likelihood ratios (PLR and NLR) were calculated in order to evaluate the performance of each test to detect malignancy.

RESULTS: Sixty-one specimens had complete sets of stains, yielding 49 and 12 specimens from pancreatic adenocarcinomas and benign pancreatic lesions due to

pancreatitis, respectively. Cytology alone had sensitivity and specificity of 41% and 100% to detect malignancy, respectively. In 46% of the specimens, routine cytology alone was deemed indeterminate. The addition of either p53 or Ki-67 increased the sensitivity to 51% and 53%, respectively, with perfect specificity, PPV and PLR (100%, 100% and infinite). Both stains in combination increased the sensitivity to 57%. While additional staining with CEA and CA19-9 further increased the sensitivity to 86%, the specificity, PPV and PLR were significantly reduced (at minimum 42%, 84% and 1, respectively). Markers in all combinations performed poorly as a negative test (NPV 26% to 47%, and NLR 0.27 and 0.70).

CONCLUSION: Immunohistochemical staining for p53 and Ki-67 can improve the sensitivity of EUS-FNA to diagnose pancreatic adenocarcinoma.

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Key words: Endoscopic ultrasound; Fine needle aspiration; Pancreatic cancer; p53; Ki-67; Immunohistochemistry

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INTRODUCTION

Pancreatic cancer is the fourth highest cause of cancer death in the United States. The overall 5-year survival rate is less than 5%, although early detection and curative resection can improve the survival rate to 20%^[1]. Endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) has high specificity for malignancy in a solid pancreatic lesion, but sensitivity varies from 70% to 90%^[2-14]. This is due to suboptimal sampling and cases of indeterminate cytology. It is difficult to distinguish well-differentiated cancers from reactive and benign cytologic changes. In such cases, tumor marker detection *via* immunohistochemistry (IH) may facilitate diagnosis. In this study, the diagnostic utility of p53, Ki-67, carcinoembryonic antigen (CEA), and CA 19-9 were assessed. The goal was to increase the sensitivity of EUS-FNA to diagnose malignancy without compromising specificity.

MATERIALS AND METHODS

Patients

Patients who underwent EUS-FNA in the years 2002 to 2008 at Harbor-UCLA Medical Center were retrospectively identified, and details were analyzed for demographic characteristics, presenting clinical features, laboratory data, imaging, and cytology results by chart review. Final diagnosis was established from: (1) tissue diagnosis consistent with malignancy; (2) imaging studies which included computed tomography (CT), magnetic resonance imaging (MRI), and EUS; and (3) clinical follow-up, including telephone calls, for at least 1 year.

Specimen acquisition and preparation

EUS-FNA was performed by 1 of 3 experienced endoscopists with a 22-gauge needle (Medi-Globe Inc or Wilson-Cook Inc) averaging 4 to 5 passes per session. The aspirate was immediately smeared onto a glass slide and fixed in 95% ethanol, and then sent to a cytopathologist for cytologic analysis. The residual material was fixed in 10% neutral buffered zinc formalin and embedded in paraffin for preservation in a cell block for IH labeling. Four micron sections of cell block were cut and deparaffinized, and two sections each were used for labeling with p53 (1:50; DO-7, Zymed), Ki-67 (1:50; MIB-1, DAKO), polyclonal CEA (1:50; Zymed), and CA19-9 (1:50; DAKO). Slides were pre-treated with 3% hydrogen peroxide in 100% methanol to block endogenous peroxidase activity and facilitate tissue permeability. The p53 and Ki-67 sections underwent further processing with heat for antigen retrieval: incubation EDTA solution (pH 8.0) at 100°C, followed by boiling water for 20-30 min. The reaction system was detected using the Signet-streptavidin peroxidase system, for one hour at 37°C. Finally, the reaction was developed with 0.5% diaminobenzidine in 0.05 mol/L Tris buffer, pH 7.4, containing 0.5% hydrogen peroxide, rinsed in tap

water, counterstained with hematoxylin, dehydrated, cleared in xylene, and mounted in permanent cover slip medium.

Cytopathology

Cytologic smears and tissue cell blocks obtained by EUS-FNA were reviewed by a cytopathologist who was blinded to the final diagnoses. For conventional cytology, the specimens were reported respectively as benign, indeterminate (atypical or suspicious for malignancy), or malignant. For IH, a positive result for malignancy was based on the following criteria for each stain: intense staining of pleomorphic nuclei for p53 (Figure 1A) and Ki-67 (Figure 1B), with additional criteria for greater than 50% of population stained for the latter, and intense and diffuse cytoplasmic staining by CA19-9 and polyclonal CEA.

Data analysis

Continuous variables were expressed as median and range, and the Mann-Whitney U test was used to determine the statistical significance of differences between two groups. Fisher's exact test was used to determine statistical significance for categorical variables. A *P* value less than 0.05 was regarded as significant for both tests. To determine the discrimination of findings from cytology and IH, the sensitivity, specificity, positive and negative predictive values (PPV and NPV), and positive and negative likelihood ratios (PLR and NLR) were calculated, each alone or in various combinations. Only specimens with complete sets of stains were included. Repeat samples were included. For the purposes of this study, atypical and suspicious specimens were deemed negative for malignancy.

RESULTS

A total of 61 specimens with complete set of stains were identified, from 12 benign and 49 malignant cases on final diagnosis. Benign pancreatic lesions were due to chronic pancreatitis; of these, 3 cases were of autoimmune etiology. Malignant masses were due to pancreatic adenocarcinoma in all 49 cases. The patient demographic information, lesion location, and laboratory information on initial presentation are summarized in Table 1. Total bilirubin and CA19-9 levels were significantly higher in patients with cancer, but no other differences were detected.

Table 2 summarizes the positive staining results for the tumor markers p53, Ki-67, CEA and CA19-9, alone or in selected parallel combinations, according to their final diagnosis and cytologic findings. The sensitivity, specificity, PPV, NPV, PLR, and NLR with regard to the detection of pancreatic malignancy were calculated for cytology and each tumor marker, alone or in various combinations. Table 3 summarizes these results when applied to all the EUS-FNA specimens in selected combinations.

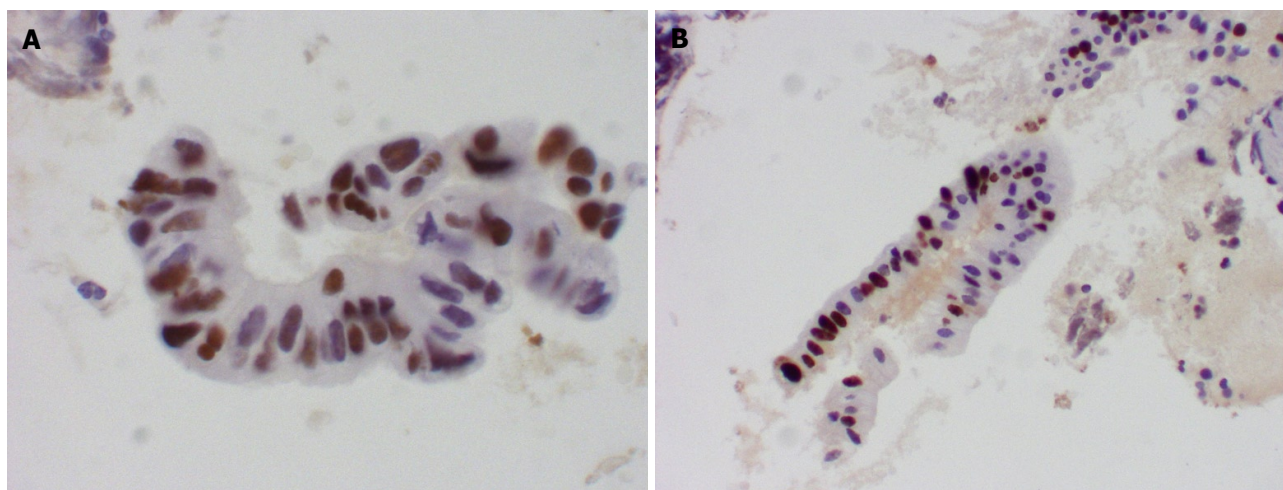


Figure 1 Immunoreactivity in pancreatic adenocarcinoma, counterstained with hematoxylin (blue), at 436 × magnification. A: Positive p53 staining of the pleomorphic nucleus (brown). B: Positive Ki-67 nuclear staining (brown), at greater than or equal to 50% of total cluster of cells.

Table 1 Patient data for those with pancreatic lesions definitively diagnosed as benign or malignant (not all laboratory data were available for each patient)

	Malignant (n = 49)	Benign (n = 12)
Age [median (range)]	61 (34-88)	60 (15-73)
Sex (M:F)	27:22:00	8:04
Location of pancreatic lesion		
Head/uncinate process	39	10
Body	9	2
Tail	1	0
Serum enzymes [median (range)]		
Total bilirubin ^a (mg/dL)	3 (1-28)	1 (0-5)
AST (U/L)	63 (11-603)	35 (13-182)
ALT (U/L)	74 (5-694)	33 (13-167)
ALP (U/L)	211 (54-1307)	136 (59-1409)
Amylase (U/L)	94 (15-182)	80 (11-285)
Lipase (U/L)	26 (13-199)	29 (16-95)
Serum tumor markers [median (range)]		
CEA (ng/mL)	5 (1-403)	3 (0-32)
CA19-9 ^a (U/mL)	339 (0-9929)	28 (0-1507)

^aP < 0.05, benign group compared with malignant. ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; CEA: carcinoembryonic antigen.

Routine cytology correctly diagnosed 20 of 49 cases to be malignant, with 8 false negatives. Twenty-eight specimens had indeterminate cytology (atypical or suspicious for malignancy), 46% of the total. They constituted 43% and 58% of malignant and benign specimens, respectively. With the assumption that the atypical and suspicious cases were benign, the sensitivity and specificity were 41% and 100%, respectively. If the suspicious specimens were deemed malignant, which proved to be so in our particular study, the sensitivity increased to 49%.

Parallel addition of each p53 or Ki-67 to cytology

increased the sensitivity to diagnose malignancy by 10% and 12%, respectively. Both stains in combination further increased the sensitivity by 16%, to 57%. Two false negatives and 6 indeterminate cases were correctly diagnosed to be malignant. The specificity, PPV and PLR remained perfect (100%, 100% and infinite).

On the other hand, while addition of CEA and CA19-9 increased the sensitivity to 86%, their utility was compromised by their poor specificity. The high false positive rates at 58% and 25%, respectively, were due to indiscriminate staining of specimens with either indeterminate or benign cytology.

Table 4 summarizes the diagnostic yield for the same tests when applied only to EUS-FNA specimens that were found to be either benign or indeterminate on cytology (therefore only in specimens with non-malignant cytology). The overall trend was preserved.

DISCUSSION

EUS-FNA of the pancreas has an excellent specificity for cancer diagnosis, but its sensitivity is tempered by cases of suboptimal sampling and indeterminate cytology. In this study, routine cytology had sensitivity of 41%, which is much lower than previously reported (usual range 70% to 90%^[2-14]). This was due to higher prevalence of indeterminate cytology at 46%, versus 4% to 26% reported in other studies^[2-14]. The discrepancy is possibly due to instances of differing cytologic criteria having been applied to different patient populations. In agreement with this study, a retrospective study of 74 EUS-FNA specimens at another southern California hospital (Torrance Memorial Medical Center, Torrance CA) also had high incidence rate of indeterminate cytologies at 39%, yielding in a sensitivity and specificity of 52% and 100%, respectively^[15]. (Procedures were performed by the endoscopist-coauthor VE, and slide evaluations

Table 2 Distribution of positive tumor marker stains, alone or in select parallel combinations, according to cytology and final diagnosis

Malignant (<i>n</i> = 49)				Final diagnosis cytology	Benign (<i>n</i> = 12)			
Benign (<i>n</i> = 8)	Atypical (<i>n</i> = 17)	Suspicious (<i>n</i> = 4)	Malignant (<i>n</i> = 20)		Benign (<i>n</i> = 5)	Atypical (<i>n</i> = 7)	Suspicious (<i>n</i> = 0)	Malignant (<i>n</i> = 0)
1	4	0	9	p53	0	0	0	0
2	4	0	14	Ki-67	0	0	0	0
6	8	1	15	CEA	4	3	0	0
4	10	0	13	CA19-9	1	2	0	0
2	6	0	16	p53:Ki-67	0	0	0	0
6	12	1	17	p53:Ki-67:CEA	4	3	0	0
5	14	0	19	p53:Ki-67:CA19-9	1	2	0	0
6	15	1	19	All 4 stains	4	3	0	0

CEA: carcinoembryonic antigen.

Table 3 Diagnostic accuracy of routine cytology and immunohistochemistry, alone or in selected combinations, in detection of pancreatic cancer *via* endoscopic ultrasound-guided fine needle aspiration

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	PLR	NLR
Cytology	41	100	100	29	¥	0.59
p53 + cytology	51	100	100	33	¥	0.49
Ki-67 + cytology	53	100	100	34	¥	0.47
CEA + cytology	71	42	83	26	1	0.7
CA19-9 + cytology	69	75	92	38	3	0.41
p53+Ki-67 + cytology	57	100	100	36	¥	0.43
p53+Ki-67 + CEA+cytology	80	42	85	33	1	0.49
p53+Ki-67 + CA19-9 + cytology	80	75	93	47	3	0.27
All 4 stains + cytology	86	42	86	42	1	0.34

CEA: carcinoembryonic antigen; IH: immunohistochemistry; NLR: negative likelihood ratio; NPV: negative predictive value; PLR: positive likelihood ratio; PPV: positive predictive value.

Table 4 Diagnostic accuracy of immunohistochemistry, alone or in selected combinations, as applied only to cytologically benign, atypical and suspicious specimens

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	PLR	NLR
p53	17	100	100	33	¥	0.83
Ki-67	21	100	100	34	¥	0.79
CEA	52	42	68	26	1	1.16
CA19-9	48	75	82	38	2	0.69
p53 + Ki-67	28	100	100	36	¥	0.72
p53 + Ki-67 + CEA	66	42	73	33	1	0.83
p53 + Ki-67 + CA19-9	66	75	86	47	3	0.46
All 4 stains	76	42	76	42	1	0.58

CEA: carcinoembryonic antigen; IH: immunohistochemistry; NLR: negative likelihood ratio; NPV: negative predictive value; PLR: positive likelihood ratio; PPV: positive predictive value.

were done by pathologists at various University of California Medical Centers). These differences further emphasize the need for assessment beyond routine aspirate cytology.

This study shows that the detection of molecular tumor markers can increase the sensitivity of EUS-FNA to diagnose pancreatic malignancy, though this is tempered by variable reductions in their specificity. IH was utilized for their detection, as more advanced tests such as DNA mutation analysis, digital image analysis (DIA)

and fluorescence *in situ* hybridization (FISH) are not routinely available, are much more expensive, need special preparation that sometimes necessitate larger samples, and have longer processing time.

By staining specimens with either benign or atypical cytology, the combination of both p53 and Ki-67 increased the pancreatic EUS-FNA sensitivity by 16%, and proved to be “pathognomonic” for malignancy with an infinite PLR. Of relevance, the abnormalities in both antigens have been correlated to the late stage of what is

now believed to be a step-wise progression from normal pancreatic epithelium to pancreatic intraepithelial neoplasia (PanIN), and then to frank adenocarcinoma^[16,17].

Ki-67 is a proliferation antigen present in all phases of the cell cycle, except the resting phase^[18]. The labeling index is directly correlated to the PanIN grade^[17,19], and clear quantitative differences in labeling can be demonstrated between malignant and benign pancreatic lesions, most notably at higher cutoff points^[20]. Findings from this study are consistent.

The tumor suppressor p53 controls cell cycle progression, differentiation and apoptosis^[21,22]. It is inactivated largely *via* mutation, usually resulting in nuclear accumulation and positive IH stain^[23-25]. The p53 abnormality is detected in 50% to 90% of pancreatic adenocarcinomas^[23-31], and in their precursor, PanIN, the positive staining is directly correlated to the grade^[17,29-31]. Our findings agree with previous studies on EUS-FNA of pancreas, which showed that the addition of p53 IH or DNA analysis resulted in a modest sensitivity gain^[12-14]. Two notable differences were observed in comparison to other studies that have included the surgical specimen. First, in contrast to this study, false positive p53 stainings in benign pancreatic lesions were demonstrated in previous studies at rates ranging from 3% to about 10%^[12,13,26,27,31-33]. Indeed, wild-type p53 over-expression can be inducible under certain benign conditions, such as during inflammation in response to TNF-alpha^[34]. Second, this study showed a lower rate of p53 IH positivity in malignant cases: 29% versus the usual range of 50% to 70% in other EUS-FNA and biopsy series^[13,14,32]. There are multiple explanations for these discrepancies, such as the criteria used for positive p53 staining, but the most likely reasons are the relatively small patient group size and the over-representation of specimens with indeterminate cytology in our study.

CEA and CA19-9 staining resulted in non-significant PLRs due to dramatic reductions in specificity. Non-specific cytoplasmic staining with either CEA or CA19-9 has previously been demonstrated in both the normal and inflamed pancreatic ductal epithelium^[35-37].

Performance was poor for all the markers when used as indicators for the absence of malignancy (alone or in various parallel combinations of up to four markers) as demonstrated by low NPVs and non-significant NLRs. This was due to suboptimal sampling, as well as the inherent limitations of IH to completely correlate marker accumulation and staining for their defect/mutation^[23-25]. Therefore, a negative test still necessitates further diagnostic measures to determine the presence or absence of malignancy.

There are a number of limitations to our study. First, not all patients had the “gold standard” - a surgical biopsy or autopsy - for definitive diagnosis. Second, only a limited number of specimens from benign pancreatic lesions were included in this study. Third, although not by design, non-ductal cancers and other neoplasias were not included in this study. This limits the scope of applica-

bility. Fourth, in cytologically benign specimens that were later correctly diagnosed as malignant by either p53 or Ki-67 staining, two arguable scenarios exist: (1) that they were truly cases of missed cytologic diagnosis; or (2) that they were suboptimal samplings with false positive staining. It is beyond the scope of this study to distinguish between them. In conclusion, the addition of tumor marker staining by IH to routine cytology can increase the diagnostic yield of pancreatic EUS-FNA. In particular, staining for both p53 and Ki-67 gave the best overall performance and appears promising for future large prospective and studies.

COMMENTS

Background

Tissue sampling *via* endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) has high specificity for the determination of malignancy of solid pancreatic lesions. But conventional cytologic examination alone can lead to either missed or ambiguous cases due to instances of suboptimal sampling and indeterminate cytology. This leads to delayed diagnosis and therapy, along with increased cost, mortality and morbidity, resulting from the necessity for further diagnostic procedures.

Research frontiers

Molecular changes associated with carcinogenesis may help to diagnose malignancy of a pancreatic lesion where routine cytologic examination of EUS-FNA samples alone is inadequate. Four well-characterized tumor markers detected by immunohistochemistry, p53, Ki-67, carcinoembryonic antigen and CA19-9, were used along with routine cytology to increase the sensitivity of EUS-FNA.

Innovations and breakthroughs

This paper shows that the use of tumor markers p53 and Ki-67 can increase the sensitivity of EUS-FNA while maintaining 100% specificity, if used with select criteria. Furthermore, immunohistochemistry is a much more cost-effective approach in comparison to other more advanced cytologic/molecular techniques, such as digital image analysis, fluorescence *in situ* hybridization, or direct DNA mutation analysis *via* polymerase chain reaction.

Applications

This study shows that the addition of tumor markers detected *via* immunohistochemistry can increase the pancreatic cancer rate detection of EUS-FNA. This leads to faster diagnosis and therapy, with fewer and less costly diagnostic procedures being needed, which may ultimately lead to a decrease in morbidity and mortality associated with pancreatic lesions. Prospective studies with larger number of cases are needed for validation.

Peer reviews

This seems like a good study showing that sensitivity for pancreatic tumor detection can be increased from 41% to 57% using p53 and Ki-67 staining on EUS-guided FNA specimens. The author appropriately admits to loss of specificity with use of multiple stains. This is an interesting twist on current technology and should be published.

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