

Serum Macrophage Inhibitory Cytokine 1 as a Marker of Pancreatic and Other Periampullary Cancers

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ABSTRACT

Purpose: Patients with pancreatic ductal adenocarcinoma usually present with advanced-stage disease and a dismal prognosis. One effective strategy likely to improve the morbidity and mortality from pancreatic cancer would be the identification of accurate, noninvasive diagnostic markers that would enable earlier diagnosis of symptomatic patients and earlier detection of cancer in asymptomatic individuals at high risk for developing pancreatic cancer. In this study, we evaluated serum macrophage inhibitory cytokine-1 (MIC-1) as a marker of pancreatic cancer.

Experimental Design: MIC-1 expression in primary pancreatic cancers, intraductal papillary mucinous neoplasms, and pancreatic cancer cell lines was determined using the National Center for Biotechnology Information serial analysis of gene expression database, oligonucleotide microarrays analysis, *in situ* hybridization, and immunohistochemistry. Serum MIC-1 levels were determined by ELISA in 80 patients with pancreatic adenocarcinomas, in 30 patients with ampullary and cholangiocellular carcinomas, in 42 patients with benign pancreatic tumors, in 76 patients with chronic pancreatitis, and in 97 healthy control subjects. The diagnostic performance of serum MIC-1 as a

marker of pancreatic cancer was compared with that of serum CA19–9.

Results: Oligonucleotide microarray and serial analysis of gene expression data demonstrated that MIC-1 RNA levels were higher in primary pancreatic cancers, intraductal papillary mucinous neoplasms, and pancreatic cancer cell lines than in nonneoplastic pancreatic ductal epithelium. MIC-1 expression was localized to the malignant epithelium in pancreatic adenocarcinomas by *in situ* hybridization. MIC-1 protein was expressed in 14 of 16 primary pancreatic adenocarcinomas (88%) by immunohistochemistry and was also expressed in some pancreata affected by pancreatitis but not in normal pancreas. Serum MIC-1 levels were significantly higher in patients with pancreatic ductal adenocarcinoma (mean \pm SD, 2428 \pm 2324 pg/ml) and in patients with ampullary and cholangiocellular carcinomas (2123 \pm 2387 pg/ml) than in those with benign pancreatic neoplasms (940 \pm 469 pg/ml), chronic pancreatitis (1364 \pm 1236 pg/ml), or in healthy controls (546 \pm 262 pg/ml). An elevated serum MIC-1 (defined as 2 SD above the mean for healthy controls) performed as well as CA19–9 (area under the receiver operating characteristic curve, 0.81 and 0.77, respectively), and the combination of MIC-1 and CA19–9 significantly improved diagnostic accuracy ($P < 0.05$; area under the receiver operating characteristic curve, 0.87; sensitivity, 70%; specificity, 85%).

Conclusion: Serum MIC-1 measurement can aid in the diagnosis of pancreatic adenocarcinoma.

INTRODUCTION

In contrast to most other gastrointestinal malignancies, the overall 5-year survival after a diagnosis of pancreatic adenocarcinoma remains abysmal (1). This exceptionally poor prognosis is attributed in part to the advanced stage of disease at which most patients with pancreatic cancer present; only ~15% of patients are operable at presentation, and the remaining 85% of patients have little chance of cure (2). Those patients with operable disease can achieve 5-year survival rates of 15–40% with pancreaticoduodenectomy (3). These statistics suggest that among patients with pancreatic cancer, early diagnosis could improve prognosis. Unfortunately, the nonsurgical diagnosis of pancreatic cancer is difficult because existing tumor markers are not sufficiently specific to reliably differentiate benign from malignant disease. Furthermore, imaging and endoscopy are less effective at diagnosing small, surgically resectable cancers than large, unresectable lesions. Finally, even when a pancreatic lesion is identified, cytologic diagnosis can be difficult, often requiring multiple investigations (4).

In addition to the difficulties in diagnosing patients with symptoms indicative of pancreatic cancer, there is a need to identify pancreatic neoplasia in individuals at high risk for developing pancreatic cancer, such as those with a strong family

Received 9/4/03; revised 12/15/03; accepted 12/19/03.

Grant support: The Mildred-Scheel-Stiftung, Deutsche Krebshilfe, National Cancer Institute Specialized Programs of Research Excellence Grant CA62924, the Lustgarten Foundation for Pancreatic Cancer Research, National Health and Medical Research Council, and New South Wales Health Research and Development Infrastructure.

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history (4–7) or in patients with familial cancer syndromes such as the Peutz-Jeghers syndrome (5, 8). Early experience with screening high-risk populations using endoscopic ultrasound has been encouraging (6). Currently used markers of pancreatic cancer such as CA19–9 have poor discriminatory value, especially in small cancers. Noninvasive diagnostic tests such as serum markers are needed to facilitate the early diagnosis and detection of pancreatic adenocarcinomas in both average-risk and high-risk populations.

Gene expression profiling studies (9–14) using serial analysis of gene expression and microarray technology have proven valuable tools in the discovery of new candidate cancer biomarkers. Among the proteins identified as overexpressed in pancreatic cancer compared with normal pancreas are S100A4, prostate stem cell antigen, mesothelin, and tissue inhibitor of metalloproteinase 1 (14–17). Some of these markers have diagnostic potential as serum markers or as immunohistochemical predictors of cancer that could be applied to cytologic specimens (15, 16).

One marker that has been identified through global gene expression profiling as overexpressed in colorectal and other cancers is macrophage inhibitory cytokine 1 (MIC-1; Ref. 18). MIC-1 is a distant member of the transforming growth factor β superfamily originally identified in the setting of macrophage activation (19). Additionally, it is known as placental transforming growth factor β (20), prostate-derived factor (21), growth/differentiation factor 15/MIC-1 (22), and placental bone morphogenetic protein (23). In addition to being overexpressed in several cancer types, MIC-1 may have anticancer functions. A number of authors have demonstrated *in vivo* and *in vitro* effects of MIC-1 on tumor growth and/or apoptosis (24). The MIC-1 promoter region is a target for p53; p53 expression increases MIC-1 expression (25–27) and this is demonstrable not only *in vitro* but also *in vivo* (28). MIC-1 is also up-regulated in colonic carcinoma cell lines treated with nonsteroidal anti-inflammatory drugs (24, 29). More recent studies have also suggested a role for MIC-1 in tumor invasion (30).

We developed a sensitive immunoassay for MIC-1 (31) and have used this assay to demonstrate elevated serum MIC-1 levels in patients with colon, breast, and prostate cancers (32, 33). These data led us to investigate the role of MIC-1 as a serum marker for pancreatic cancer.

MATERIALS AND METHODS

Serum and Tissue Samples. A total of 326 preoperative serum samples were obtained from patients undergoing pancreaticoduodenectomy at the Johns Hopkins Medical Institutions, including 80 patients with pancreatic adenocarcinoma, 20 with ampullary adenocarcinoma, 10 with cholangiocarcinoma, and 42 with other pancreatic neoplasms [27 with pancreatic serous or mucinous cystadenomas, 9 with pancreatic neuroendocrine tumors, and 6 with intraductal papillary mucinous neoplasms (IPMNs)]. Sera were also obtained from 77 patients with chronic pancreatitis and from 97 healthy volunteers who were undergoing screening colonoscopy. Information on the tumor-node-metastasis status was available for 72 of the 80 patients with pancreatic adenocarcinoma ($T_1N_0M_x$, $n = 1$; $T_2N_1M_x$, $n = 3$; $T_3N_0M_x$, $n = 2$; $T_3N_1M_x$, $n = 56$; $T_4N_0M_x$, $n = 1$; metastatic

disease, $n = 9$). The mean age \pm SD and the sex profile (male:female) of each patient group was 66.4 ± 11.4 years, 45 males:35 females for patients with pancreatic adenocarcinoma; 63.1 ± 12.2 years, 19:11 for patients with ampullary and cholangiocellular carcinoma; 61.4 ± 14.4 years, 14:28 for patients with other pancreatic tumors; and 53.6 ± 15.4 years, 51:25 for patients with chronic pancreatitis. *In situ* hybridization and immunohistochemistry were performed on paraffin-embedded, formalin-fixed tissue sections of surgically resected pancreatic adenocarcinomas and nonneoplastic pancreas obtained from the Surgical Pathology archives of the Johns Hopkins Medical Institutions. All of the samples were collected with approval from the Johns Hopkins Committee for Clinical Investigation.

MIC-1 Gene Expression Analysis. The SAGEmap public database⁶ was queried to identify MIC-1 mRNA expression in pancreatic cancer cell lines and in normal pancreatic ductal cell lines.

MIC-1 RNA expression was determined from Affymetrix U133A oligonucleotide microarray data generated for 12 IPMNs, 5 pancreatic cancer cell lines (AsPc1, CFPAC1, Hs766T, MiaPaca, and Panc-1), 5 microdissected nonneoplastic pancreatic ductal tissue samples, and a normal pancreatic ductal epithelium cell line (HPDE6) using methods described previously (11).

MIC-1 *In Situ* Hybridization. Preparation of digoxigenin-labeled riboprobes and nonradioactive *in situ* hybridization were performed based on protocols published previously (34, 35). For the *in situ* hybridization of formalin-fixed tissue, sections were deparaffinized for 5 min followed by hydration in graded ethanols for 5 min each. Next, sections were digested with proteinase K (15 μ g/ml) at 37°C for 40 min. This was followed by hybridization overnight with MIC-1 riboprobe in mRNA hybridization buffer at 45°C. Subsequently, sections were washed in $2\times$ SSC at 55°C, followed by incubation with a 1:35 dilution of RNase A mixture (Ambion, Austin, TX) in 10 mmol/liter Tris, 500 mmol/liter NaCl, and 1 mmol/liter EDTA (pH 7.5) for 1 h at 37°C. Next, slides were washed twice in $2\times$ SSC/50% formamide at 50°C, followed by one wash at $0.08\times$ SSC, also at 50°C. Signal amplification was achieved by incubation of sections with biotinyl-tyramide, followed by secondary streptavidin complex. The final signal was developed with diaminobenzidine chromagen (GenPoint kit; DAKO, Carpinteria, CA).

MIC-1 Immunohistochemistry. Immunohistochemistry was carried out on formalin-fixed, paraffin-embedded tissue from 16 matched pairs of pancreatic adenocarcinoma and nonmalignant pancreatic tissue from the same patients in a tissue microarray format. Sections were dehydrated in xylene and graded ethanols, boiled for 30 min in 0.01 M citrate buffer (pH 6), and washed three times for 2 min in dH₂O. Endogenous peroxidases were blocked with 3% H₂O₂/PBS and, after washing three times for 10 min with 0.5% Triton-X/PBS, sections were blocked with 2% BSA (w/v), 2% fetal bovine serum (v/v) for 1 h and then incubated with sheep anti-MIC-1 antiserum

⁶ Internet address: <http://www.ncbi.nlm.nih.gov/SAGE>.

(233-BP, 1:2000) at 4°C for 16 h. After washing three times with 0.5% Triton-X/PBS, sections were incubated with peroxidase-conjugated donkey antisheep antibody (Jackson ImmunoResearch Inc., West Grove, PA) for 1 h, washed three times for 10 min with 0.5% Triton-X/PBS, and then developed with diaminobenzidine (DAKO Corp.), counterstained with hematoxylin, dehydrated, and mounted.

MIC-1 ELISA. The MIC-1 sandwich ELISA was performed as described previously (18, 31, 36). The mouse monoclonal anti-hMIC-1 antibody, 26G6H6, was used for antigen capture, and the sheep anti-MIC-1 antibody, 233B-P, was used for antigen detection. Data on the performance characteristics of the sandwich ELISA have been published elsewhere (31, 37).

CA 19-9 ELISA. A total of 25 μ l of serum were analyzed with a commercially available ELISA kit (MucinPC/CA19-9 ELISA; α Diagnostic Int., San Antonio, TX) according to the manufacturer's recommendations. CA19-9 was determined in 314 of the 326 samples used in this study.

Statistical Analysis. The major statistical end point in this study was receiver operating characteristic analysis of CA-19 and MIC-1 for the diagnosis of pancreatic adenocarcinoma. The area under the receiver operating characteristic curve

(AUC) was calculated for each marker, and bootstrap bias-corrected accelerated confidence intervals were computed using version 2.5 of AccuROC for Windows 95/98/NT Software (Accumetric Corp., Montreal, Quebec, Canada). The comparison of these correlated single-marker curves was made with AccuROC, using the method of DeLong *et al.* (38). To compare the predictive value of a model using both markers to the use of CA-19 alone, a bootstrap approach was taken (39). Bootstrap samples were used to estimate the AUC for each model and their difference. The sample SD of the difference is the bootstrap estimate of its SE. A paired Student's *t* test was used to determine significance. Means were compared using simple linear regression models after log transformation of the data when necessary. Means and SDs are reported on the natural scale. Statistical computations were performed using AccuROC or the SAS system (SAS institute, Cary, NC) and all of the *P*s reported are two sided.

RESULTS

MIC-1 Gene Expression Analysis. An online query of the SAGEmap public database⁶ revealed a relative overexpres-

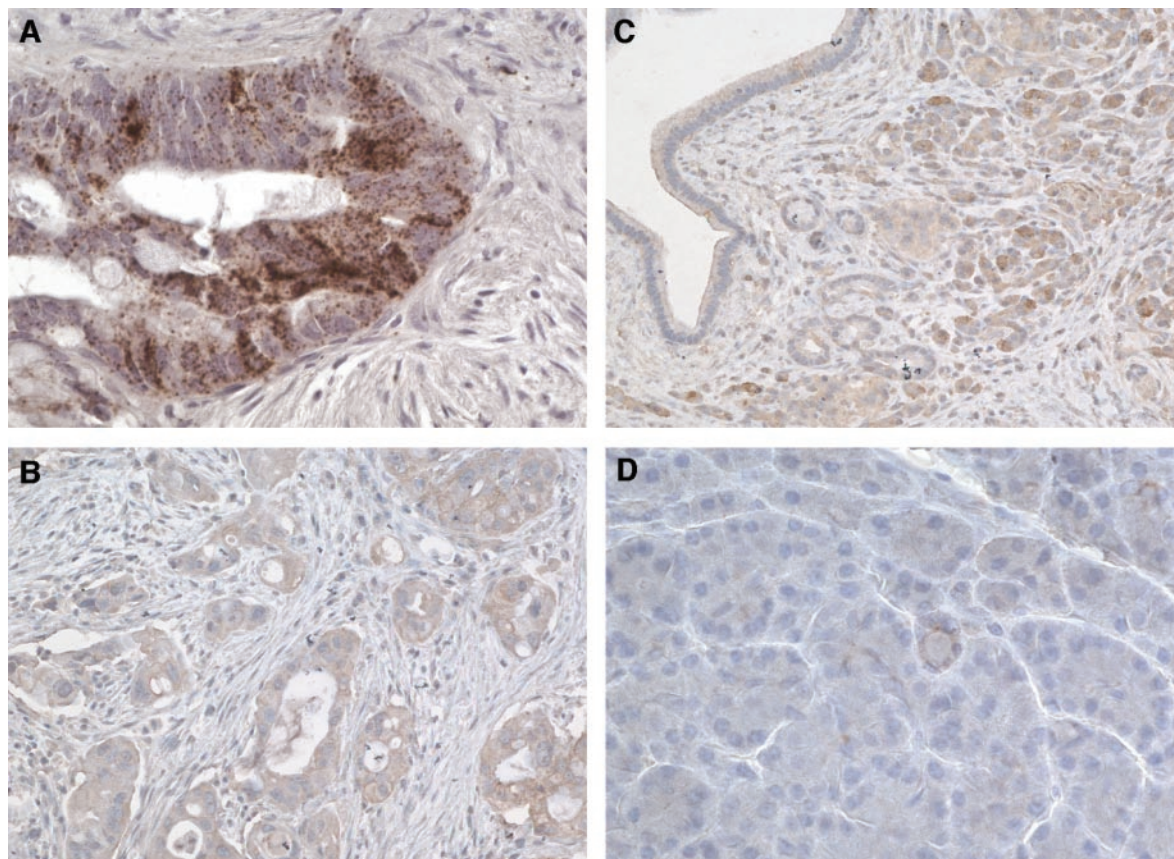


Fig. 1 Macrophage inhibitory cytokine-1 (MIC-1) *in situ* hybridization and immunohistochemistry. **A**, nonradioactive *in situ* hybridization for MIC-1. A specific mRNA signal is seen in the malignant epithelia of a pancreatic adenocarcinoma (magnification $\times 100$). **B**, MIC-1 immunohistochemistry. MIC-1 protein is expressed in the cytoplasm of pancreatic adenocarcinoma cells (magnification $\times 64$). **C**, MIC-1 immunohistochemistry. Areas of chronic pancreatitis also show MIC-1 protein expression (magnification $\times 64$). **D**, MIC-1 immunohistochemistry. Normal pancreatic acini only exhibit faint background staining (magnification $\times 100$).

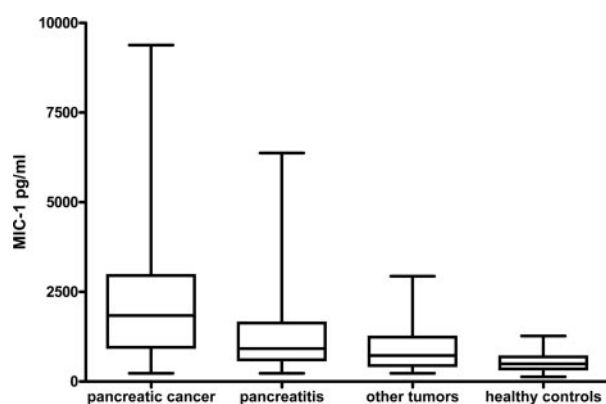


Fig. 2 Boxplot of macrophage inhibitory cytokine-1 (MIC-1) serum levels for pancreatic adenocarcinoma, pancreatitis, other pancreatic tumors, and normal controls; bars, \pm SD.

sion of MIC-1 in pancreatic adenocarcinoma cell lines Panc-1 and CAPAN-1 (1003 and 210 tags/million) compared with normal pancreatic ductal cell lines H126 and HX (30 and 93 tags/million).

Gene expression of MIC-1 using oligonucleotide microarrays was also analyzed in 5 pancreatic cancer cell lines and in 1 normal pancreatic ductal cell line, revealing a 5.5-fold overexpression (mean signal intensities \pm SD: cancer cell lines, 474.8 ± 282.2 ; HPDE cell line, 85.1). Furthermore, overexpression of MIC-1 mRNA was found by gene expression analysis in a comparison of 12 IPMNs and 5 microdissected normal pancreatic ductal tissue specimens (mean signal intensities \pm SD: IPMNs, 1564.4 ± 1196.77 ; normal ducts, 489.3 ± 220.2 ; $P = 0.01$, Student's t test).

MIC-1 Nonradioactive *in Situ* Hybridization and Immunohistochemistry. MIC-1 tissue expression was investigated additionally by nonradioactive *in situ* hybridization on paraffin-embedded, formalin-fixed, surgically resected pancreatic adenocarcinoma tissue. A strong mRNA signal was observed in the neoplastic epithelial cells (Fig. 1A). No MIC-1-specific labeling was seen in stromal cells or within normal pancreatic acinar tissue. A riboprobe specific for regenerating islet-derived 1 α was used as a positive control for tissue RNA quality. The regenerating islet-derived 1 α probe demonstrated positive labeling of atrophic pancreatic acini in areas of chronic inflammation, as described previously (Ref. 40; data not shown).

MIC-1 immunohistochemistry revealed positive staining in 14 of 16 pancreatic adenocarcinoma samples. The labeling was localized to the cytoplasm of malignant cells (Fig. 1B). Of the 16 matched pancreatic control tissues, 7 samples showed MIC-1 protein expression. Labeling in these cases was restricted to atrophic acini, metaplastic ducts, and pancreatic islets in areas of chronic pancreatitis (Fig. 1C). Normal pancreatic acini exhibited no specific staining (Fig. 1D).

MIC-1 Serum Levels. For analysis purposes, the serum samples were subdivided into pancreatic ductal adenocarcinoma, ampullary and cholangiocellular carcinomas, other pancreatic neoplasms, chronic pancreatitis, and healthy control groups. The respective mean MIC-1 levels \pm SD in each group

were patients with pancreatic adenocarcinomas, 2428 ± 2324 pg/ml; patients with ampullary adenocarcinomas, 2168 ± 2757 pg/ml; patients with cholangiocarcinomas, 2032 ± 1516 pg/ml; and patients with other pancreatic tumors (cystadenomas, 750 ± 62 pg/ml; neuroendocrine tumors, 1568 ± 983 ; and IPMNs, 850 ± 100 pg/ml) 940 ± 469 pg/ml; patients with chronic pancreatitis, 1364 ± 1236 pg/ml; and healthy controls, 546 ± 262 pg/ml (Fig. 2; Table 1). The mean MIC-1 level among all of the patients with cancer that were analyzed was 2345 ± 2334 pg/ml, and the corresponding mean for all of the patients without cancer was 905 ± 803 pg/ml. MIC-1 levels were significantly higher in patients with pancreatic cancer than in those with other pancreatic neoplasms, those with chronic pancreatitis, and in the healthy control group ($P < 0.05$, Student's t test). MIC-1 levels were also significantly higher in patients with other periampullary cancers compared with both patients with benign pancreatic disease and healthy controls.

To determine the sensitivity of elevated MIC-1 as a diagnostic test, we used a cutoff of 1070 pg/ml, a level 2 SDs above the mean MIC-1 level in the healthy control group. Using this cutoff, we found that 57 of 80 patients with pancreatic adenocarcinoma (80%) had an elevated MIC-1 level, as well as 10 of 20 patients with ampullary adenocarcinoma (50%) and 7 of 10 patients with cholangiocarcinoma (70%), corresponding to an overall prevalence of 67% of patients (74 of 110) with any periampullary cancer. These proportions of patients with an elevated MIC-1 level were compared with 32 of 77 patients with chronic pancreatitis (42%), 3 of 27 patients with pancreatic cystadenomas (11%), 6 of 9 patients with a pancreatic neuroendocrine tumor (67%), 2 of 6 with an IPMN (33%; 1 of the IPMNs displayed an area of carcinoma *in situ*), and 4 of 97 of healthy controls (4%). In total, 47 of 216 patients without cancer (22%) had an elevation of MIC-1 >1070 pg/ml.

Comparison of the Diagnostic Accuracy of Serum MIC-1 and CA19-9. To evaluate the performance of serum MIC-1 as a diagnostic test, receiver operating characteristic curves were obtained by plotting sensitivity *versus* specificity. The respective AUC as a measure of diagnostic performance for MIC-1 (cutoff 1070 pg/ml) in the discrimination of pancreatic adenocarcinoma *versus* noncancer samples was 0.81 (95% confidence interval, 0.75–0.86; Fig. 3A). With this cutoff MIC-1 had a sensitivity of 0.71 and specificity of 0.78 in discriminating patients with pancreatic adenocarcinoma

Table 1 Mean serum MIC-1^a \pm SD for the diagnostic subgroups

Diagnosis	N	Serum MIC-1	SD
Pancreatic adenocarcinoma	80	2428	± 2324
Ampullary adenocarcinoma	20	2168	± 2757
Cholangiocellular carcinoma	10	2032	± 1516
Chronic pancreatitis	77	1364	± 1236
Cystadenoma	27	750	± 62
Neuroendocrine tumor	9	1568	± 983
IPMNs	6	850	± 100
Healthy controls	97	546	± 262
All adenocarcinoma	110	2345	± 2334
All other pancreatic tumors	42	940	± 469
All noncancer	216	905	± 883

^a MIC-1 = macrophage inhibitory cytokine 1; IPMN = intraductal papillary mucinous neoplasm.

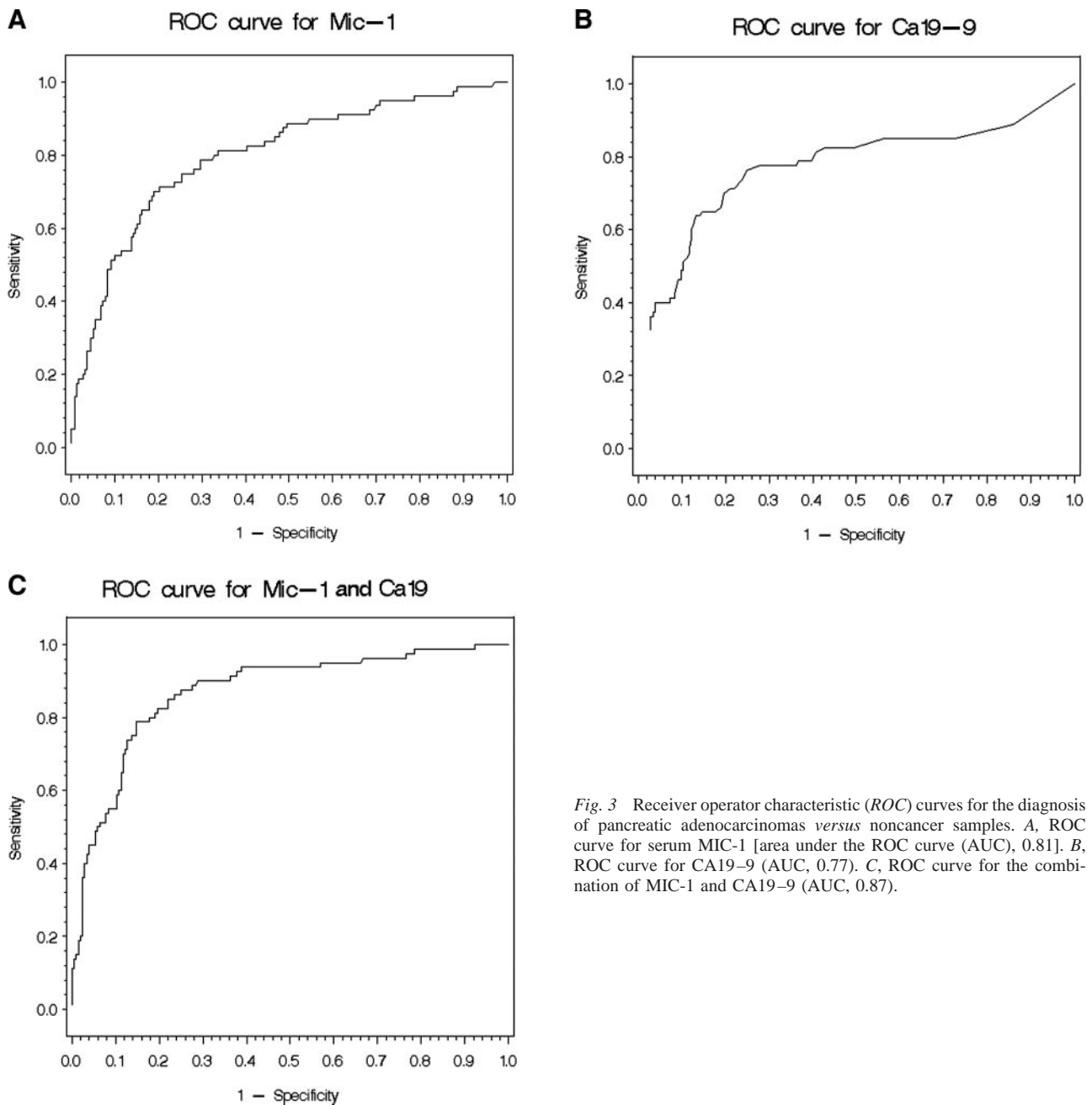


Fig. 3 Receiver operator characteristic (ROC) curves for the diagnosis of pancreatic adenocarcinomas versus noncancer samples. *A*, ROC curve for serum MIC-1 [area under the ROC curve (AUC), 0.81]. *B*, ROC curve for CA19-9 (AUC, 0.77). *C*, ROC curve for the combination of MIC-1 and CA19-9 (AUC, 0.87).

from the patients without cancer that were analyzed in this study. For differentiating all of the patients with periampullary adenocarcinomas (pancreatic ductal, ampullary, and cholangiocellular) from those patients without cancer, MIC-1 had an AUC of 0.79 (95% confidence interval, 0.73–0.84). We also compared the respective diagnostic performances of MIC-1 and CA19-9 in 314 patients for whom we had determined both MIC-1 and CA19-9 levels. CA19-9, at a cutoff of 70 units/ml, had a similar AUC to that of MIC-1 (0.77; 95% confidence interval, 0.69–0.83), with a sensitivity of 0.59 and specificity of 0.88 for differentiating patients with

pancreatic adenocarcinoma from patients without cancer (Fig. 3*B*). The difference in the MIC-1 and CA19-9 AUCs was not statistically significant ($P = 0.32$, Student's *t* test). We also determined the diagnostic utility of combining serum MIC-1 with CA19-9 measurements (Fig. 3*C*). The diagnostic performance achieved by a combination of MIC-1 and CA19-9 was significantly better than that using CA19-9 alone (AUC, 0.87; 95% confidence interval, 0.82–0.92; $P < 0.001$), with a sensitivity of 70% and specificity of 85% using an MIC-1 cutoff of 3000 pg/ml, or a sensitivity of 89% and specificity of 72% using an MIC-1 cutoff of 1070 pg/ml.

Correlation of Serum MIC-1 with Clinicopathologic Features. There was no significant association between MIC-1 levels and tumor size. Likewise, among patients with cancer there was no correlation between lymph node status and MIC-1 level. Patients with elevated preoperative serum bilirubin had significantly higher MIC-1 levels ($P = 0.005$, Student's t test; means 1658 and 2759 pg/ml for normal/elevated bilirubin groups).

DISCUSSION

In this study we found that in a population of patients with periampullary pancreatic disease, serum MIC-1 performs as well as CA19-9 in distinguishing patients with pancreatic and other periampullary adenocarcinomas from patients with non-cancerous lesions of the periampullary region. Furthermore, combining both MIC-1 and CA19-9 yielded greater diagnostic accuracy (sensitivity, 70%; specificity, 85%) than CA19-9 alone. The diagnostic performance of MIC-1 is noteworthy considering that our pancreatic cancer population was mostly limited to patients with small, resectable pancreatic adenocarcinomas and that we used a disease control group consisting of patients with a wide variety of periampullary diseases. Indeed, discrimination of patients with nonmalignant periampullary disease from those with pancreatic malignancies and identification of patients with small resectable pancreatic adenocarcinomas are the two major hurdles any new diagnostic marker of pancreatic cancer must overcome.

MIC-1 behaved similarly in serum from patients with ampullary and cholangiocellular carcinomas as in patients with pancreatic ductal adenocarcinoma. Although we included a modest number of patients with such cancers ($n = 30$), these results, with the results of recent studies demonstrating elevated levels of MIC-1 in patients with other malignancies (41), suggest that MIC-1 may be a useful marker of many epithelial neoplasms.

We found no correlation between tumor size or lymph node status and MIC-1 levels. This is contrary to the strong association of serum MIC-1 with tumor stage and survival seen in colorectal neoplasms (41). The determination of the MIC-1 genotype could be of prognostic value, as the D and H alleles have been linked to decreasing relapse-free survival and decreasing survival time, respectively (41).

A combination of oligonucleotide microarray, serial analysis of gene expression, *in situ* hybridization, and immunohistochemistry data demonstrate that MIC-1 is overexpressed in pancreatic cancer tissues. MIC-1 expression was localized to the cytoplasm of pancreatic cancer cells, but also could be found in areas of the pancreas affected by pancreatitis.

Because tissue levels of MIC-1 RNA were elevated in IPMNs but were elevated only minimally in the serum of these patients it is possible that MIC-1 levels may not rise significantly in the serum of such patients until they have developed an invasive cancer. If this is the case, MIC-1 may also be useful for distinguishing IPMNs with an associated invasive adenocarcinoma from IPMNs without an associated invasive carcinoma. Because MIC-1 levels also were not elevated significantly in patients with noncancerous neoplastic pancreatic cysts, MIC-1 levels may be similarly useful in differentiating cystic lesions

with an invasive cancer from nonmalignant cysts. However, when a patient presents with a pancreatic lesion the more common diagnostic question is whether the patient has a pancreatic neoplasm, and determination of serum MIC-1 levels may prove useful in specific circumstances. For example, in patients with comorbid disease, the distinction between a relatively benign cystic neoplasm and an invasive cancer can spare the patient the need for surgical exploration.

Like many tumor markers of pancreatic cancer, MIC-1 has only modest ability to distinguish between pancreatic cancer and pancreatitis. Additional studies are needed to determine whether the diagnostic utility of MIC-1 could be improved further by combining MIC-1 not only with CA19-9 but also with other serum markers that have shown some promise as pancreatic cancer markers, such as hepatocarcinoma-intestine-pancreas/pancreatitis-associated protein I and tissue inhibitor of metalloproteinase 1 (14, 42).

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