

Short Communication

Evaluation of Osteopontin as Biomarker for Pancreatic Adenocarcinoma

Jens Koopmann,¹ Neal S. Fedarko,³ Alka Jain,³ Anirban Maitra,¹ Christine Iacobuzio-Donahue,¹ Ayman Rahman,¹ Ralph H. Hruban,¹ Charles J. Yeo,² and Michael Goggins¹

¹Departments of Pathology and ²Surgery, Johns Hopkins Medical Institutions, Baltimore, MD and ³Division of Geriatrics, Department of Medicine, Johns Hopkins Bayview Medical Center, Baltimore, MD

Abstract

Objective: Pancreatic adenocarcinoma is a deadly disease with an overall 5-year patient survival of less than 5%. This dismal prognosis of pancreatic cancer is largely due to the advanced stage of the disease at presentation. If pancreatic cancer could be diagnosed more readily and accurately using serum markers, patient survival could theoretically be improved by enabling more patients to avail of surgical resection. One candidate tumor marker recently identified by global gene expression analysis of pancreatic cancer is the secreted glycoprophosphoprotein osteopontin (OPN). In this study, we evaluate OPN as a serum marker of pancreatic adenocarcinoma. **Methods:** *In situ* hybridization for OPN was performed on a pancreatic adenocarcinoma tissue microarray. Serum OPN levels were determined in preoperative sera from 50 patients with pancreatic cancer and 22 healthy control individuals by competitive ELISA. **Results:** *In situ* hybridization for OPN performed on a tissue microarray revealed strong

OPN mRNA signal in tumor-infiltrating macrophages in 8 of 14 pancreatic adenocarcinomas. In contrast, OPN expression was not seen in the pancreatic cancer cells themselves, nor was it seen in normal pancreatic tissue or in the macrophages distant from the infiltrating cancer. Serum OPN levels, as measured by ELISA, were elevated in the sera of 50 patients with resectable pancreatic adenocarcinoma compared to 22 healthy control individuals (mean \pm SD for OPN was 482 ± 170 ng/ml and 204 ± 65 ng/ml, respectively; $P < 0.001$). Using a cutoff level of 2 SD above the mean for healthy individuals, elevated OPN had sensitivity of 80% and specificity of 97% for pancreatic cancer. In contrast, only 62% of these patients with resectable pancreatic cancer had elevated CA19-9. **Conclusion:** Serum OPN may have utility as a diagnostic marker in patients with pancreatic cancer. (Cancer Epidemiol Biomarkers Prev 2004;13(3):487–491)

Introduction

Currently, the most effective treatment for pancreatic ductal adenocarcinoma is surgical resection, but the disease is usually unresectable at diagnosis and only 10–15% of patients are candidates for potentially curative resection (1). Those patients that can undergo pancreatic resection can achieve a median 5-year survival of 20–30% (2). The clinical diagnosis of pancreatic cancer is often difficult because existing tumor markers such as CA19-9 are not sufficiently specific to reliably differentiate benign from malignant disease and also because cytological detection of cancer

requires invasive and often repeated investigation (3). Although the accuracy of imaging and endoscopic approaches continues to improve, it is often difficult to identify individuals with small surgically resectable cancer (1, 4). In addition, there are currently no serum markers that can be used for screening of patients at high risk of developing pancreatic cancer such as those with a strong family history of the disease (3, 5–7) or patients with familial cancer syndromes such as the Peutz-Jeghers syndrome (5, 8). Thus, a sensitive and specific serum biomarker that could diagnose early stage pancreatic adenocarcinoma would have the potential to improve the prognosis of pancreatic cancer by increasing the number of individuals detected with resectable disease.

One powerful approach for identifying new cancer markers is through the use of RNA-based global gene expression strategies. Recently, several groups have obtained global gene expression profiles of pancreatic adenocarcinomas using a variety of approaches (9–13). Among the many genes identified as differentially overexpressed, only a minority encode for known secreted proteins, which are attractive candidates to

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Requests for reprints: Michael Goggins, Department of Pathology, The Johns Hopkins Medical Institutions, Room 632, Ross Building, 720 Rutland Avenue, Baltimore, MD 21205-2196. Phone: (410) 955-3511; Fax: (410) 614-0671. E-mail: mgoggins@jhmi.edu

use as serum biomarkers (9, 10). These genes include *Lipocalin*, urokinase type plasminogen activator (*u-PA*), tissue inhibitor of metalloproteinase 1 (*TIMP-1*), and osteopontin (*OPN*). *Lipocalin*, *u-PA*, and *TIMP-1* have already been reported previously as overexpressed in pancreatic cancer, underscoring the usefulness of the gene expression-based approach for identifying candidate diagnostic markers (13–15). In this study, we report the results of an evaluation of *OPN* as a serum marker for pancreatic adenocarcinoma.

Materials and Methods

Serum and Tissue Samples. Preoperative serum samples from 50 patients undergoing pancreaticoduodenectomy for pancreatic adenocarcinoma at the Johns Hopkins Medical Institutions were collected as part of an ongoing observational study. Serum from 22 healthy control individuals was obtained from the Johns Hopkins Bayview Medical Center General Clinical Research Center. The mean age of the tumor group was 66.5 years (range 47–87) and 43.5 for the healthy control group (range 21–62). Sera were stored at -80°C . Formalin-fixed paraffin-embedded tissue blocks from patients with pancreatic adenocarcinoma were obtained from the archives of the Department of Pathology at the Johns Hopkins Hospital. Tissue microarrays were constructed as previously described (16). All of these analyses were performed with approval of the Johns Hopkins Medical Institutions Institutional Review Board.

Nonradioactive *in Situ* Hybridization. Preparation of digoxigenin-labeled sense and antisense riboprobes and nonradioactive *in situ* hybridization were performed based on previously published protocol (17). For the *in situ* hybridization of formalin-fixed tissue, sections were deparaffinized in xylene for 5 min followed by hydration in graded ethanols for 5 min each. Next, sections were digested with proteinase K (15 $\mu\text{g}/\text{ml}$) at 37°C for 40 min. This was followed by hybridization overnight with antisense or sense riboprobes (300 ng/ml concentration) in mRNA hybridization buffer at 45°C . Subsequently, sections were washed in $2\times$ SSC at 45°C , followed by incubation with a 1/35 dilution of RNase A cocktail (Ambion, Austin, TX) in 10 mM Tris, 500 mM NaCl, 1 mM EDTA (pH 7.5), for 1 h at 37°C . Next, slides were washed twice in $2\times$ SSC/50% formamide at 55°C , followed by one wash at $0.08\times$ SSC also at 55°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 Corp., Carpinteria, CA).

CD68 Immunohistochemistry. The CD68 antibody, clone KP1 (DAKO), was used as primary antibody (1:4000) on paraffin-embedded, formalin-fixed pancreatic adenocarcinoma tissue. Slides were heated at 90°C for 20 min in citrate buffer (Vector Lab., Burlingame, CA) and then processed using a 3,3'-diaminobenzidine (DAB)-based detection system (Ventana Corp., Tucson, AZ). Counterstaining was done with hematoxylin.

OPN Serum ELISA. A modified competitive serum ELISA procedure for *OPN* was developed by one of the

authors (N.F.) and performed as described previously (18). In brief, serum samples were diluted 1:10 in a 50% formamide-40 mM phosphate buffer (pH 7.4) and were reduced with 2 mM DTT at 100°C for 5 min to disrupt the binding of *OPN* by complement factor H in serum. Residual reducing agent and formamide were removed by strong anion exchange column chromatography (ToyoPearl QAE resin; TosoHaas, Montgomeryville, PA). After loading the 100- μl sample, the column was washed with $6\times$ column volumes of Tris-buffered saline with Tween 20 (TBS-T) solution (50 mM Tris, 150 mM NaCl, and 0.05% Tween 20). *OPN* was eluted with TBS-T containing 1.0 M NaCl. For the competitive ELISA, Greiner high-binding plates (USA Scientific, Inc., Ocala, FL) were coated with 20 ng/well *OPN* overnight in 50 mM carbonate buffer (pH 8.0). The standard curves for *OPN* were constructed using recombinant protein. Samples and standards (100 μl volume) were incubated for 2 h with shaking at room temperature with 100 μl of a 1:100,000 of LF-124 antibody in TBS-T in polypropylene 96-well plates (USA Scientific). During those 2 h, the antigen-coated plate was blocked with TBS + 5% powdered milk. Antigen-coated plate was then rinsed three times with TBS-T, and the antibody-sample solution was added to the wells. After a second incubation for 1 h at room temperature with shaking, the plate was washed three times with TBS-T. A secondary antibody of goat anti-rabbit peroxidase-labeled antibody conjugate, human serum adsorbed (Kirkegaard & Perry, Gaithersburg, MD) at 1:2000 was then added, and the plates were incubated for 1 h. After three washes with TBS-T, substrate (TMB microwell peroxidase substrate; BioFX Laboratories, Owings Mills, MD) was added and after a final 20-min incubation, the color reaction was stopped by the addition of 25 μl of 1 N H_2SO_4 . Absorbance was read at 450 nm. A coefficient of variation of 15–20%, mainly attributable to the column purification step, has been published previously for this assay (18).

CA19-9 Serum ELISA. CA19-9 levels were measured in serum samples (25 μl) by commercially available ELISA (Alpha Diagnostics Inc., San Antonio, TX) according to the manufacturer's recommendations.

Statistical Analysis. For serum *OPN* levels, sensitivity (incidence of true positive results when the assay was applied to patients known to have pancreatic cancer) and specificity (incidence of negative results when the assay was applied to healthy subjects) were calculated. Descriptive statistical values and plots were generated using the Microsoft Excel software package. For the comparison of the mean *OPN* values for cancer and normal sera, the independent samples *t* test function of the SPSS 10.0 for Windows software package (confidence interval 95%) was applied.

Results

***In Situ* Hybridization for *OPN*.** A strong *OPN* mRNA signal was observed in tumor-infiltrating cells with morphological characteristics of monocytes/macrophages in 8 of 14 (57%) tissue cores of pancreatic adenocarcinoma (see Fig. 1). Immunohistochemical

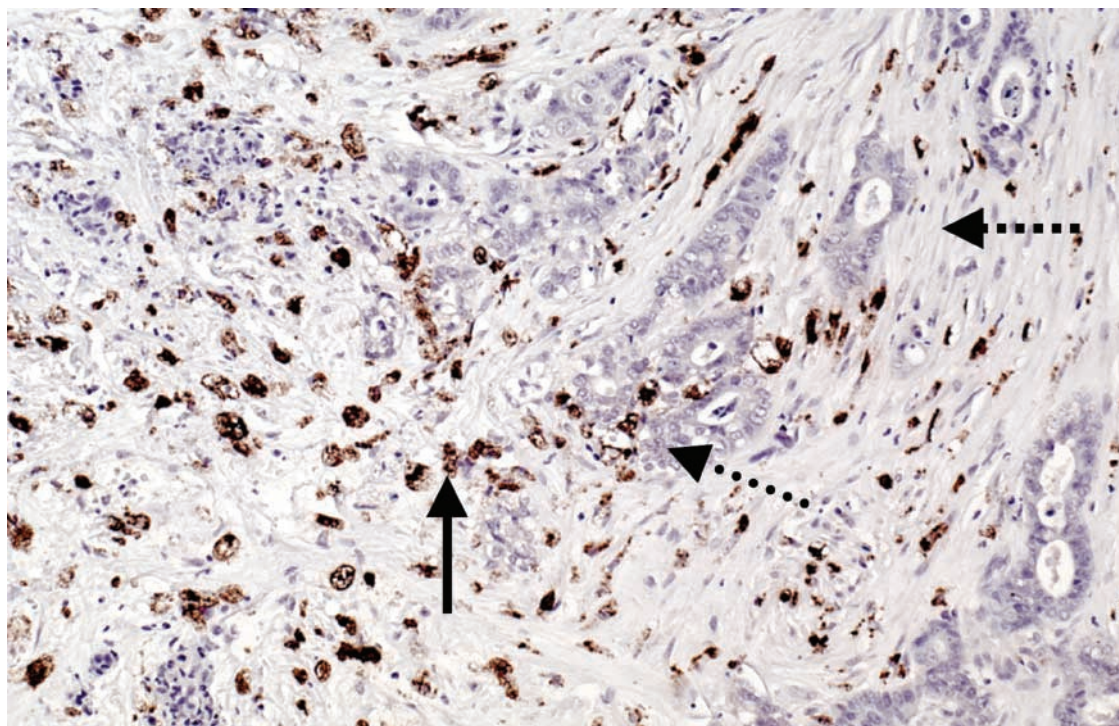


Fig. 1. *In situ* hybridization for OPN in pancreatic adenocarcinoma tissue. A strong OPN mRNA signal is seen in tumor-infiltrating macrophages (solid arrow), but not in tumor (dotted arrow) or stromal (dashed arrow) cells. Counterstaining with CD68 confirmed the macrophage origin of the infiltrating cells (data not shown).

labeling using CD68 confirmed the macrophage origin of those cells (data not shown). Interestingly, OPN-positive macrophages were restricted to the tumor and its immediate vicinity. Weak OPN labeling was seen in atrophic pancreatic ducts in areas of tumor-associated pancreatitis. No OPN mRNA signal could be demonstrated in CD68-positive macrophages in areas distant from the cancer. Also, no labeling for OPN was seen in adenocarcinoma cells, tumor stroma cells, or normal pancreatic control tissue. We also performed *in situ* hybridization on the resection cancer specimens of three patients with pancreatic adenocarcinoma who had the highest levels of serum OPN (see next subsection). A similar pattern of labeling in their pancreatic cancer was observed.

OPN and CA19-9 Serum ELISA. Mean serum OPN levels were elevated ~2.5-fold in sera from patients with pancreatic adenocarcinoma compared to normal healthy controls ($P < 0.001$, independent samples t test, see Fig. 2). The mean OPN serum level for pancreatic adenocarcinoma patients was 482 ± 170 ng/ml (range 192–919) and 204 ± 65 ng/ml (range 69–370 ng/ml) for normal controls. We used a cutoff level of 334 ng/ml for serum OPN, which corresponded to 2 SD above the mean OPN in the healthy controls. Forty of the 50 pancreatic cancer samples (80%) showed OPN levels above this cutoff, and 21 of the 22 (95%) healthy samples below. This corresponded to a sensitivity and specificity of elevated OPN for pancreatic cancer of 80% and 97%, respectively; and a 98% positive predictive value and 76% negative predictive value. For the sensitivity to reach 100%, a corresponding specific-

ity of 41% was obtained. We also measured CA19-9 levels in 48 of the 50 patients with pancreatic adenocarcinoma. Using the more stringent cutoff value of 70 units/ml (1), 30 of 48 (62%) of patients with pancreatic adenocarcinoma had elevated CA19-9. At a less stringent cutoff level for CA19-9 of 37 units/ml (1), 38 of 48 (79%) of the pancreatic cancer sera contained elevated CA19-9. Interestingly, all cancer sera

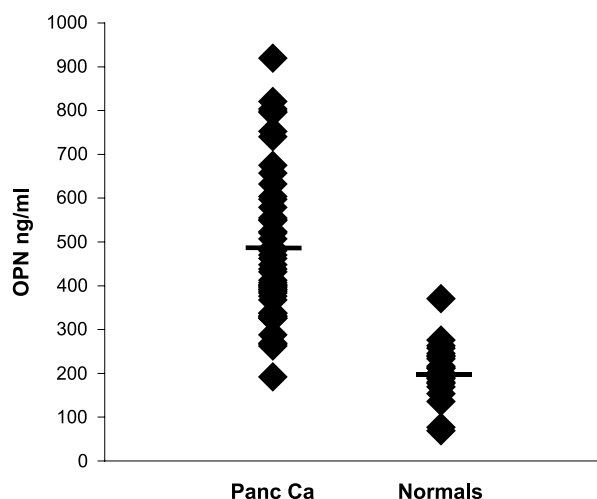


Fig. 2. Serum OPN levels in pancreatic adenocarcinoma and normal controls. Serum OPN was significantly elevated in sera from pancreatic adenocarcinoma patients compared to normal control individuals ($P < 0.001$). Mean/SD for OPN was 482 ± 170 ng/ml and 204 ± 65 ng/ml, respectively.

($n = 10$), which were not identified correctly by CA19-9 (cutoff 37 units/ml), were correctly classified by their elevated OPN levels.

Thus, the sensitivity of a combination of CA19-9 and OPN (either CA19-9 > 70 units/ml or OPN > 334 ng/ml) for the detection of cancer patients was 100%. Serum OPN levels in our patient group showed no correlation with tumor size or tumors-nodes-metastasis (TNM) status (data not shown).

Discussion

In this study, we demonstrate that serum OPN is a potential new serum biomarker for pancreatic adenocarcinoma. Our pancreatic cancer patient population included only patients with resectable disease, the most appropriate patient group for testing the utility of new serum markers (4). Previous studies have demonstrated comparable elevations of OPN serum levels in patients with ovarian, colon, breast, and prostate cancer (18, 19). Given these promising results, it would be useful to know if serum OPN is elevated in patients with non-neoplastic pancreatic diseases. The degree of elevation of OPN in our study (~2.5-fold) is comparable with previously reported serum OPN levels in lung, breast, and prostate cancer (18) and plasma OPN levels in ovarian cancer (19). In this study, OPN outperformed CA19-9, the current standard biomarker for pancreatic cancer. OPN's sensitivity (80%) compares favorably with the sensitivity for CA19-9 (62% using the 70 units/ml cutoff, or 79% using the 37 units/ml cutoff). Similarly, the 97% specificity for serum OPN among healthy controls also compares favorably to the specificity of CA19-9 (1).

OPN is a glycoposphoprotein of M_r 55,000–80,000, normally produced by osteoblasts, arterial smooth muscle cells, various epithelia, activated T cells and macrophages and secreted into most body fluids (20). As a member of the small integrin binding N-linked glycoproteins (SIBLING) family, OPN is able to bind to extracellular matrix proteins through its Arg-Gly-Asp (RGD)-motif and signal transducing receptors (integrin receptors, CD44v) via distinct binding sites (21, 22). OPN functions as a signaling molecule, either in the soluble form or as immobilized cell adhesion protein. The functions most likely related to tumorigenesis include facilitation of anchorage-independent growth in transformed cells (23), stimulation of migration and invasion (23), binding and activation of matrix metalloproteinases (24), protection from apoptosis (25), potential evasion of complement-mediated attack by binding of OPN to serum complement factor H (26), enhancement of metastatic ability (27), and direct stimulation of cancer cell proliferation and progression (28). In clinical studies, OPN has been associated with decreased survival in cancer patients (29), increased metastatic potential (30), and advanced disease stage (31). OPN was one of the genes we previously identified as overexpressed (7.9-fold) in pancreatic cancer through global gene expression profiling using the Affymetrix platform (9). Because of the secreted nature of the OPN protein and the known involvement in tumorigenesis, we hypothesized that OPN would be a potential diagnostic marker

for patients with pancreatic adenocarcinoma. A similar approach had previously identified OPN as a plasma biomarker in patients with ovarian carcinoma (19). By *in situ* hybridization, OPN was expressed in tumor-infiltrating macrophages in 8 of 14 (57%) examined tissue cores and was not detected in normal pancreatic acinar or ductal epithelial cells. This finding is in accordance with the previously observed pattern of OPN expression restricted to tumor-infiltrating macrophages in other gastrointestinal cancers entities and in contrast to OPN expression by the adenocarcinoma cells proper in endometrial and kidney carcinomas (32). We conclude that the primary source for OPN in pancreatic adenocarcinoma tissue originates from activated macrophages infiltrating the tumor.

The ultimate diagnostic utility of serum OPN can only be determined if future studies demonstrate that it has high specificity for cancer as opposed to non-neoplastic diseases. Because OPN is expressed in macrophages, it is likely that serum OPN will be significantly increased in patients with chronic granulomatous inflammation and possibly in patients with other inflammatory conditions as well as in patients with other carcinomas. Because patients with pancreatic inflammation are often distinguishable from those with cancer with other clinical assays, such a limitation might not prevent OPN from being used as a marker of pancreatic or other cancers. Because OPN mRNA expression has also been demonstrated in chronic pancreatitis tissues (33), it is possible that pancreatitis will also induce elevations of OPN. If so, OPN may help improve the identification of pancreatic cancer if other markers can be used to rule out the presence of pancreatitis. OPN may also have a diagnostic utility as a part of a marker panel that includes other markers such as CA19-9, TIMP-1 (13), and hepatocarcinoma-intestine-pancreas, pancreatitis-associated protein (HIP/PAP) (34, 35), as well as for follow-up of patients undergoing treatment for pancreatic cancer in whom other serum markers such as CA19-9 are uninformative (36).

In conclusion, we have demonstrated that serum OPN has a diagnostic potential as a biomarker for pancreatic adenocarcinoma.

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