Identifying Markers for Pancreatic Cancer by Gene Expression Analysis[1]

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Abstract

To begin to identify new tumor markers, we recently performed a systematic study of gene expression in cancers of the colon and pancreas. Of the 45,000 genes identified, 183 were found to be expressed at significantly elevated levels in pancreatic cancer. One of the genes was *tissue inhibitor of metalloproteinase type I* (*TIMP-1*), which encodes a secreted protein. Analysis of TIMP-1 serum levels revealed significant increases in pancreatic cancer patients, but TIMP-1 by itself was inadequate as a serum marker for cancer. However, a combination of individually suboptimal markers (TIMP-1, CA19-9, and carcinoembryonic antigen) detected 60% of 85 patients with pancreatic cancers in a highly specific manner. These results suggest that a systematic analysis of gene expression can reveal novel serum markers and that individually suboptimal markers can be combined to yield higher sensitivity and specificity.

Introduction

One key to effective cancer treatment is early detection. In many cancers, including those of the pancreas, current methods for early detection are inadequate. Pancreatic cancer affects about 27,000 individuals in the United States each year (1) and is usually asymptomatic until it has reached an advanced stage (2, 3). Therefore, simple and cost-effective tests for early detection of pancreatic cancer might prove valuable in decreasing mortality from this disease. Here, we present an approach for detecting pancreatic cancers through serum assays.

Serum tumor markers are substances that are produced by tumor cells and are secreted or released into the bloodstream (4). The measurement of such markers is noninvasive and is relatively simple and inexpensive to perform, compared to those methods that are invasive or require special instrumentation and personnel. The ideal tumor marker for any cancer type has yet to be found, although several are being used clinically or actively investigated. Prostate-specific antigen is currently in widespread use for detecting prostate cancers, although it is not cancer specific (5, 6). Serum α-fetoprotein is useful in the diagnosis of hepatocellular carcinoma, but it also increases in benign liver disease (7). Therefore, it is commonly used with abdominal ultrasonography for screening purposes (8). CEA[3] is used as a prognostic marker for a variety of tumor types, but it lacks the sensitivity and specificity that are required for a presymptomatic marker (9, 10). Of the serum tumor markers that are available for pancreatic cancers, CA19-9 is currently the best, but limited specificity and sensitivity has restricted its application (11, 12). Additionally, 5% of the general population are genotypically Lewis $a-b-$ and cannot synthesize the carbohydrate antigen recognized by the CA19-9 antibody (13).

Most tumor markers have been found serendipitously or through monoclonal antibodies generated by immunizing mice with human tumor cells. For example, CA19-9 was originally discovered through monoclonal antibody studies of colorectal cancer cell lines (14) and was later found to be more specific for pancreatic cancer (11). To develop new markers with potentially higher sensitivity and specificity, we have used a molecular biology-based approach. We have used SAGE (15–17) to investigate expression patterns in specific cancer types, as compared to normal tissues or other cancers. Of the differentially expressed genes, those that are abundantly expressed in cancers and encode secreted or cell surface proteins can be further evaluated as potential serum tumor markers. Here, we report the first application of this approach, using pancreatic cancer as a prototype.

Materials and Methods

Tumor and Normal Tissue Sample Collection. Primary pancreatic adenocarcinomas were collected from surgically resected pancreas and frozen. Ten specimens, judged to contain 45–65% tumor cells by histopathological examination, were used for molecular analyses. Normal kidney, pancreas, skeletal muscle, breast, blood, prostate, and thymus tissues were collected from biopsies of the corresponding tissues. Normal neoplastic colon tissue were collected as described previously (17). The resectability of pancreatic cancers could be determined through medical records in 69 of the 85 patients evaluated.

Northern Blot Analysis. Total RNA was isolated from blood and tissues and used in Northern analyses as described previ-

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[3] The abbreviations used are: CEA, carcinoembryonic antigen; SAGE, serial analysis of gene expression; TIMP-1, tissue inhibitor of metalloproteinase type I.

ously (18). Expressed sequence tag clone 265330 contains the entire coding sequence of TIMP-1 and was obtained from Genome Systems, Inc. The coding region of TIMP-1 was PCR-amplified from this plasmid with the aid of M13 forward and reverse primers, and the PCR product was radioactively labeled (19) and used as probe.

ELISA. Preoperative serum samples were collected from patients with pancreatic cancer (n = 85), breast cancer (n = 64), and normal controls (n = 98) and stored at −80°C. Serum measurements were performed using the TIMP-1 ELISA system (RPN 2611; Amersham Life Science) or the CEA and CA 19-9 assays (Chiron Diagnostics), following manufacturers' protocols. Briefly, all three assays are sandwich immunoassays using two purified antibodies against the same antigen. The first antibody is used to capture the corresponding antigen, and the second antibody is coupled to a chemical reagent to give a signal. A direct relationship exists between the concentration of the antigen in the serum and the relative signal detected by the assays. Values from 5 μl of serum were measured and compared to standard curves constructed with purified antigen.

Results

TIMP-1 Expression Is Elevated in Pancreatic Tumors. As reported previously, SAGE tag libraries were generated from two primary pancreatic cancers, two pancreatic cell lines, two colorectal cancers, two colorectal cell lines, and two samples of normal colorectal epithelium (17). A total of 303,000 transcript tags, approximately 60,000 from each tissue type, were analyzed and found to correspond to over 45,000 different genes. Further analysis revealed 183 transcripts that were elevated in both primary pancreatic tumors and pancreatic cancer cell lines (available over the internet at http://welchlink.welch.jhu.edu/~molgen-g/home.htm). One of the most prominent of these differentially expressed transcripts (H560056, tag GAGAGTGTCT), was found to be derived from the *TIMP-1* gene. This tag was identified 32 times in the 60,000 tags derived from pancreatic cancers but only 0 and 5 times in an equivalent number of tags from normal colorectal mucosa and colorectal cancer tissue, respectively.

To further confirm this differential expression, Northern blot analysis was performed. *TIMP-1* was found to be expressed at high levels in each of 10 pancreatic cancers (Fig. 1*A*). In contrast, *TIMP-1* expression was not detectable or was expressed at relatively low levels in normal tissues (Fig. 1*B*).

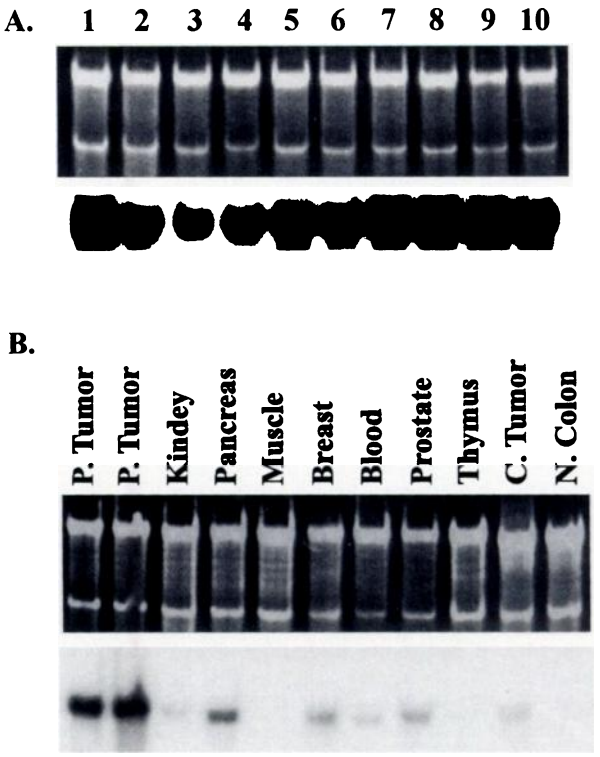

Fig. 1. TIMP-1 expression in tumor and normal tissues. *Bottom*, *TIMP-1* levels were determined by Northern blot analysis of total RNA (5 μg) isolated from 10 primary pancreatic cancers (*A*) or normal tissues (*B*). *Top*, total RNA was visualized by ethidium bromide staining. The normal tissues of origin are as indicated. *C.*, colorectal; *P.*, primary; *N.*, normal. Total RNA isolated from primary tumors 4 and 5 (*A*, *Lanes 4* and *5*) were also used as positive controls in *B* (*leftmost two lanes*).

Serum TIMP-1 Is Elevated in Pancreatic Cancer Patients. *TIMP-1* encodes a M_r 30,000 serum protein that binds to matrix metalloproteinases and inhibits their activities (20, 21). As a result, TIMP-1 circulates in blood either as a free form or as an enzyme-inhibitor complex. The use of free TIMP-1 as a serum marker has been proposed for a variety of diseases but has had limited success. For example, TIMP-1 is significantly elevated in synovial fluid from rheumatoid arthritis patients compared to normal controls, but its free serum level in these patients is only slightly elevated (185 *versus* 157.5 ng/ml; Ref. 22). Likewise, free serum TIMP-1 is only moderately elevated in patients with bladder and prostate cancer (23, 24). Recently, an ELISA system was developed that can detect both the free and complexed forms of TIMP-1 (25). This ELISA was used to evaluate the total TIMP-1 concentration in preoperative sera from pancreatic cancer patients (n = 85), breast cancer patients (n = 64) and healthy controls (n = 98). Total TIMP-1 protein concentration was found to be significantly higher in the sera of pancreatic cancer patients (1736 ± 864 ng/ml) than in those of breast cancer patients (907 ± 415 ng/ml, $P < 0.00001$) or normal controls (818 ± 347 ng/ml, $P < 0.00001$; Fig. 2*A*). Levels greater than 1550 ng/ml were observed in 43 pancreatic patients (51%) but only in four (6%) breast cancer patients and two (2%) normal controls. Furthermore, there seemed to be no correlation between serum TIMP-1 levels and tumor resectability; levels in the resectable and unresectable groups were 1687 ± 772 ng/ml (range, 503-3809 ng/ml; n = 54) and 1751 ± 931 ng/ml (range, 601-3447 ng/ml; n = 12), respectively. Hence, serum TIMP-1 levels appeared to be elevated relatively early during pancreatic cancer progression. Finally, there was no correlation between total TIMP-1 concentration and the age or gender of normal controls (date not shown), a finding consistent with previous observations (22, 26).

TIMP-1 as a Diagnostic Tumor Marker for Pancreatic Cancer. The elevation of TIMP-1 expression in pancreatic cancer patients prompted us to explore the possibility of using this protein as a tumor marker. At present, CEA is the most widely used diagnostic marker for cancer in general, and CA19-9 is the best available marker for pancreatic cancer (9–11). As shown in Fig. 2, *B* and *C*, the serum concentration of each of these markers was, on average, higher in pancreatic cancer patients than it was in healthy individuals. However, there was a large variation among the cancer patients, compromising both sensitivity and specificity. By combining the markers and using high cutoffs, we found that good specificity could be obtained while retaining reasonable sensitivity. For example, the sera from 81% of pancreatic cancer patients but only from

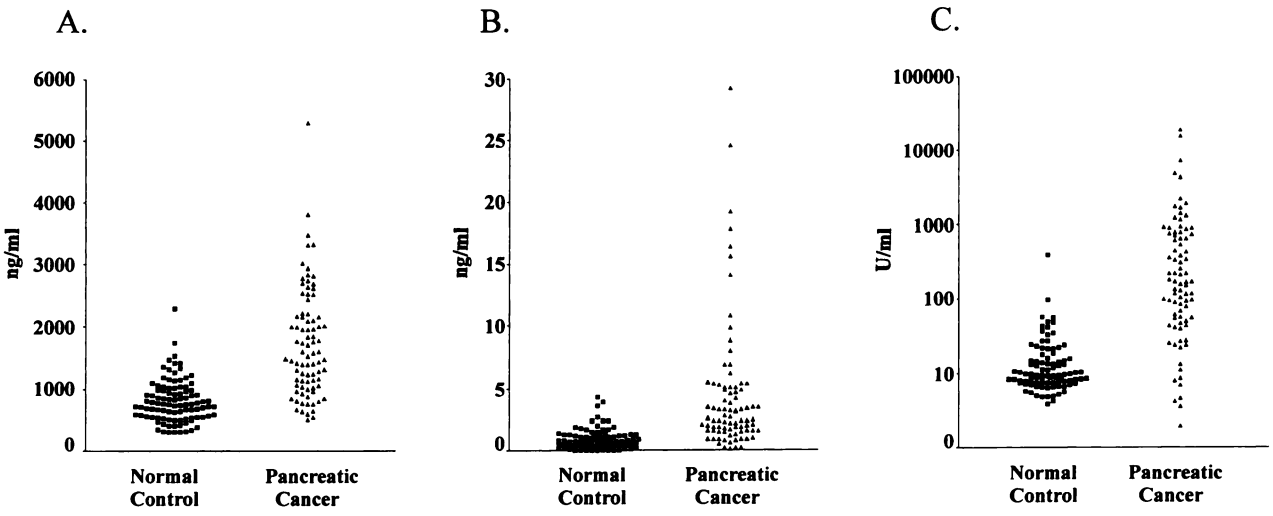

Fig. 2. Tumor marker serum levels of pancreatic cancer patients and normal controls. TIMP-1 (*A*), CEA (*B*), and CA19-9 (*C*) levels in serum from patients with pancreatic cancer (n = 85) or normal controls (n = 98) were determined as described in "Materials and Methods."

Table 1 Combinations of tumor markers can improve sensitivity and specificity

CA19-9 (units/ml)	CEA (ng/ml)	TIMP-1 (ng/ml)	Specificity (%)	Sensitivity (%)
		A. Sensitivity optimized[a]		
>136.7			99	54
	>3.3		97	41
		>1837	99	40
>136.7	>3.3	>1837	95	81
		B. Specificity optimized[b]		
>400			100	34
	>5		100	26
		>2300	100	22
>400	>5		100	48
>400		>2300	100	49
	>5	>2300	100	41
>400	>5	>2300	100	60

[a] Cutoff values were set at mean + 3 SDs of the normal controls.
[b] Cutoff values were set above the highest normal control values.

5% of healthy controls scored positive for at least one of the markers when the cutoff values for each was kept at a relatively high value (Table 1A). Although these criteria could prove useful for screening symptomatic populations, they are obviously not adequate for screening a presymptomatic population in which nearly 100% specificity is required. However, by using higher cutoff values, we were able to detect 60% of the patients with pancreatic cancer while maintaining 100% specificity (Table 1B). However, as discussed below, the clinical value of this test for screening presymptomatic population at its current state is not certain.

Discussion

What is the potential use of markers for pancreatic cancer? This cancer type carries a dismal prognosis, with over 95% of patients dying within 5 years. However, some patients with early disease survive after surgery. Unfortunately, most patients remain asymptomatic until late in the course of their disease. Presymptomatic tests for the presence of pancreatic cancers could, therefore, be of substantial use. We envision that a cost-effective and simple test to detect presymptomatic pancreatic cancers could facilitate earlier diagnosis and surgical cure in a subset of patients. Such tests, however, must meet stringent criteria if they are to be practical. Only 27,000 new cases are expected in the United States this year, and to avoid a high fraction of false positives in any generally administered test, the specificity of such a test must be extremely high. Each of the markers studied here exhibited such specificity if high cutoff values were chosen, but the sensitivity of any single test was relatively low at these cutoff values (34, 26, and 22% for CA19-9, CEA, and TIMP-1, respectively). The use of a panel including all three tests increased the sensitivity to 60% while maintaining 100% specificity. Therefore, although additional studies are obviously required, our results suggest two useful approaches for cancer detection in the future. First, they show that a panel of individually suboptimal markers may prove more helpful than any single marker. Second, they demonstrate that the evaluation of global gene expression patterns in cancers with SAGE can reveal unexpected transcripts that provide new opportunities for marker development. The integration of these two strategies is attractive in that additional markers can simply be added to the panel as they are discovered, retaining specificity through high cutoffs while expanding sensitivity. We envision that other genes identified through SAGE or other techniques might be recruited in this regard. Four other genes that are expressed at high levels in pancreatic cancers compared to other tissues or cancer types have already been identified by SAGE and confirmed by Northern blot analysis, and efforts to generate monoclonal antibodies to their encoded proteins are underway.

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