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Serum HSP70:

A Novel Biomarker for Early Detection of Pancreatic Cancer

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Abstract

Objectives—Heat shock protein 70 (HSP70) is overexpressed in human pancreatic cancer cell lines. To determine if serum HSP70 levels are elevated in patients with pancreatic cancer and can function as a biomarker for early detection of pancreatic cancer.

Methods—Study subjects were divided into 3 groups: histologically proven pancreatic cancer (PC; n = 23), chronic pancreatitis (CP; n = 12), and matched normal control subjects (C; n = 10). Serum HSP70 levels were determined using a novel immunoelectrophoresis method developed and validated by the authors. Significance of difference between the groups was analyzed with analysis of variance (ANOVA). Receiver operating characteristic (ROC) curve analysis was performed to discriminate patients with pancreatic cancer from normal controls.

Results—The mean $\pm$ SE serum HSP70 levels in the PC, CP, and C groups were 1.68 ± 0.083 ng/mL, 0.40 ± 0.057 ng/mL, and 0.04 ng/mL, respectively. Serum HSP70 levels in the PC group were significantly higher compared with either the CP or C groups ($P < 0.01$). The sensitivity and specificity of elevated serum HSP70 in the PC group was 74% and 90%, respectively.

Conclusions—Serum HSP70 levels are significantly increased in patients with pancreatic cancer and may be useful as an additional biomarker for the detection of pancreatic cancer.

Keywords

pancreas; cancer; detection; biomarker; serum; HSP70

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Pancreatic ductal adenocarcinoma is the fourth leading cause of cancer death in the United States,¹ with an estimated 35,000 deaths per year. It has the highest mortality of all the major cancers, with a 5-year survival rate of only 3% to 5% and a median survival of less than 6 months.¹ Most patients with pancreatic cancer present with advanced disease at the time of diagnosis, and only 15% to 20% of patients are candidates for potentially curative resection.² Despite attempted curative resection, most patients will eventually have recurrence both locally and systemically and ultimately die of the disease presumably because of the presence of micrometastatic disease present at the time of diagnosis.^{3–8} Therefore, there is an urgent need to develop novel markers of pancreatic cancer to facilitate early diagnosis. It has been suggested that detection of a single or group of noninvasive markers can be used to monitor the disease, select high-risk patients, and perhaps lead to prompt intervention and enhance survival of these patients.⁹

Currently, there is no reliable diagnostic test for the early detection of pancreatic adenocarcinoma. Imaging modalities such as pancreas protocol computed tomographic scan, magnetic resonance imaging, endoscopic retrograde cholangiopancreatography, and especially, endoscopic ultrasound (EUS) have significantly improved the ability to diagnose pancreatic cancers.^{10,11} However, the disease is often diagnosed at an advanced stage and owing to its retroperitoneal location in the body, lesions within the pancreas can be difficult to palpate, image, or subject to biopsy, further emphasizing the need of a noninvasive test that can detect pancreatic cancer in early stages and can easily be repeated periodically in high-risk patients.

A number of serum biomarkers have been identified for early detection of pancreatic cancer. Serum carcinoembryonic antigen (CEA), carbohydrate antigens 19-9 (CA19-9) and CA242 have been investigated individually and as combined tests for the diagnosis and prognosis of pancreatic cancer, with some encouraging results.^{12,13} Novel biomarkers such as CEA cell adhesion molecule (CEACAM1)¹⁴ and macrophage inhibitory cytokine 1 have been reported to be better than CA19-9 in detecting pancreatic cancer.¹⁵ However, the clinical use of these biomarkers remains to be determined.

Heat shock proteins (HSPs) are intracellular polypeptides, which are induced in response to a variety of stimuli and act as molecular chaperones carrying out several housekeeping functions in the cell.¹⁶ Several studies have linked serum HSPs to autoimmune diseases, gastrointestinal tract disorders, and breast as well as pancreatic cancers.^{17,18} Although the mechanism of HSP release into the serum is not completely understood, we postulated that their presence in serum may provide a novel diagnostic test for the detection of these disorders. Heat shock protein 70 is overexpressed at the messenger RNA level in pancreatic cancer cell lines¹⁹ and in human pancreatic cancer tissue.¹⁸ However, pancreatic tissue is generally not available until after surgical resection, when the disease is usually already well advanced. Measurement of HSP-70 in the serum of patients with pancreatic cancer may provide a reliable noninvasive diagnostic biomarker for early detection. Interestingly, there have been recent reports evaluating HSP27 as a serological marker for breast cancer²⁰ and pancreatic cancer²¹; however, its sensitivity for the detection of pancreatic cancer was only 62%.

Herein, we report the first study evaluating HSP70 as serological marker for pancreatic cancer based on a novel immunoelectrophoresis method developed and validated in our laboratory.²²

MATERIALS AND METHODS

This study was approved by the Institutional Review Board (IRB) of Sinai Hospital of Baltimore, Maryland. Informed consent was obtained by subjects before collection of serum samples. The study included a total of 45 patients who were divided into 3 groups: group 1, pancreatic cancer (n = 23); group 2, chronic pancreatitis (n = 12); and group 3, age-matched normal healthy controls (n = 10).

Inclusion Criteria

Group 1 (pancreatic cancer: n = 23) included men and women with established diagnosis of pancreatic cancer. Diagnosis is established with histological evidence of pancreatic adenocarcinoma on biopsy, obtained through surgery, EUS, or interventional radiology.

Group 2 (chronic pancreatitis: n = 12) included patients with prior episodes of pancreatitis, defined clinically or radiologically as calcifications on computed tomography, or dilatation of pancreatic duct on endoscopic retrograde cholangiopancreatography, now presenting with recurrent abdominal pain and elevated serum lipase/amylase levels with no evidence of pancreatic adenocarcinoma.

Group 3 (n = 10) consisted of normal healthy subjects, without any pancreatic or other systemic illnesses, and taking no medications, that were age and sex matched to the test groups.

Exclusion Criteria

Patients with clinically significant comorbid illnesses were excluded from the study. Such illnesses included hepatitis, cardiomyopathy, renal failure, myocardial infarction/ischemia, systemic lupus erythematosus, sleep apnea, pregnancy, and sepsis. Patients taking increased doses of supplemental vitamins and/or amino acids were excluded, as these can induce the HSPs. Patients who were unable to stop nonsteroidal anti-inflammatory drugs for short period of time were also excluded.

Isolation of Serum HSP

Serum HSP70 was isolated based on a novel technique previously described. The steps undertaken are summarized as follows:

HSP Isolation and Extraction From Serum

Two hundred fifty microliters of serum was incubated overnight with 12.5- μ L premade HSP 70 antibody coupled beads and shaken at 4°C overnight. Samples were suspended in 50 μ L of laemmli buffer and boiled for 8 minutes (to elute protein off the coupled beads), centrifuged, and the supernatants stored for Western blotting.

Western Blotting

An equal amount of protein was loaded onto 4% to 20% gradient polyacrylamide gel (Bio-Rad, Hercules, Calif) and electrophoresis performed at 75 V for 2 hours. Proteins were blotted onto nitrocellulose paper using mini-trans-blot cell (Bio-Rad) at 40 mA for 18 hours. Nonspecific binding sites were blocked by incubating blots with 7% nonfat dry milk in phosphate-buffered saline containing 0.1% TWEEN. Blots were then incubated monoclonal antibody for human HSP70 (Sigma Chemical, St. Louis, Mo) followed by antimouse immunoglobulin horseradish peroxidase–linked whole antibody (Amersham Pharmacia, NJ). Heat shock protein 70 bands were quantified by densitometric analysis using the Stratagene Eagle Eye II software (Stratagene, La Jolla, Calif).

Preparation of HSP70 Antibody–Coupled Protein A Sepharose Beads

Fifty microliters of HSP70 antibody (Sigma Chemicals, St Louis, Mo) was bound to 100 µg of protein A sepharose beads (Sigma Chemicals) for 2 hours with gentle rocking at room temperature. The beads were washed twice with 10 volumes (1 mL) of 0.2-mol/L sodium borate (pH, 9) by centrifuging at 3000g for 5 minutes, and resuspended in 0.2-mol/L sodium borate buffer. Enough dimethyl pimelimidate was added for a 20-mmol/L final concentration. The beads were then mixed for 30 minutes at room temperature in a shaker. The reaction was then stopped by washing the beads in 0.2-mol/L ethanolamine (pH, 8.0) and incubated for 2 hours at room temperature with gentle mixing. After the final wash, the beads were resuspended in 1-mL phosphate-buffered saline with 0.1% Merthiolate for long-term storage.

Statistical Analysis

Statistical analysis was performed using the SPSS software version 10.0. Results were calculated as median with range or mean $\pm$ SEM as appropriate. Analysis of variance, Student *t* test, and Pearson χ^2 were used to analyze for significant differences. The diagnostic performance of our test or accuracy to discriminate patients with pancreatic cancer from normal controls was evaluated using receiver operating characteristic (ROC) curve analysis.

RESULTS

The mean age of the pancreatic cancer group was 61 years (10 males and 13 females); 47.5 years (5 males and 7 females) in the chronic pancreatitis group and 54 years (6 males and 4 females) in the control group. Serum was collected from subjects in each of the 3 groups, and HSP70 was quantified using the method described. A detectable level of serum HSP70 was considered as elevated. Samples were run in triplicate, and the mean HSP70 value was used for analysis to curtail false-positive detection of serum HSP70 and convey consistency.

Mean serum HSP70 levels were quantified in all the 3 groups and are depicted in Table 1. Elevated levels of HSP70 were seen in 74% (17/23) of the patients with pancreatic cancer, 58% (7/12) of the patients with chronic pancreatitis, and only 10% (1/10) of the control patients. The magnitude of serum HSP70 elevation in the 3 groups is depicted in the scatter diagram shown in Figure 1.

The mean $\pm$ SE serum HSP70 level in patients with pancreatic adenocarcinoma (1.68 ± 0.083 ng/mL) was significantly ($P = 0.01$) higher than the control group (0.043 ng/mL; Table 2). Mean $\pm$ SE serum HSP70 level was also significantly higher in patients with chronic pancreatitis (0.40 ± 0.057 ng/mL) compared with matched control subjects ($P = 0.05$). There was a trend toward increase in serum HSP70 levels between the patients with pancreatic cancer and those with chronic pancreatitis; however, this failed to reach significance ($P = 0.065$).

The overall sensitivity and specificity of serum HSP70 for differentiating patients with pancreatic cancer from healthy controls was 74% and 90%, respectively. The positive predictive value (PPV) and negative predictive value (NPV) were 95% and 60%, respectively. Comparison of the pancreatic cancer group with the chronic pancreatitis group revealed sensitivity, specificity, PPV, and NPV of 74%, 64%, 68%, and 70%, respectively.

The accuracy of serum HSP70 to discriminate the patients with pancreatic cancer from normal controls was evaluated using ROC curve analysis. The serum HSP70 levels in the 3 groups was plotted on ROC curves, and area under the curve (AUC) was calculated as shown in Figure 2. When comparing patients with pancreatic cancer with the controls, the serum HSP70 generated a curve with an AUC of 0.7937 and a 95% confidence interval (CI) of 0.67–0.91. The comparative AUC for chronic pancreatitis versus controls was 0.6933 (95% CI, 0.48–0.90); and pancreatic cancer versus chronic pancreatitis was 0.681 (95% CI, 0.58–0.77). These data were later compared to already available ROC analysis data for other pancreatic cancer biomarkers in the literature. Receiver operating characteristic analysis proved that the accuracy of serum HSP70 to detect pancreatic cancer (AUC = 0.7937) was only slightly lower than CA19-9 (0.805) but much higher than CA 242 (0.749) or CEA (0.705).

DISCUSSION

A tumor biomarker that allows for the reliable diagnosis of pancreatic cancer has remained elusive. The US Preventative Services Task Force (USPSTF) does not currently recommend a screening program for individuals with average risk for development of pancreatic cancer.²⁶ However, high-risk patients with known inherited predisposition are encouraged to enroll in screening clinical trials evaluating the efficacy of an algorithm using magnetic resonance imaging or EUS.¹⁰ However, both these techniques are expensive, and EUS is additionally an invasive diagnostic modality. Noninvasive and cost-effective diagnostic tests are urgently needed to screen the population at risk for developing pancreatic cancer and to detect these tumors at earlier stages amenable to more effective therapy.

In the current study, we used a novel technique of measuring HSP70 in human serum and applied it to a group of patients with pancreatic carcinoma, a group with chronic pancreatitis, and to normal controls. Our data suggest clinically significant higher levels of serum HSP70 in patients with pancreatic cancer compared to normal controls (1.68 ± 0.083 vs 0.043 g/mL; $P = 0.013$). This comparative significance is all the more noteworthy because of the evolving role of HSP70 in pathogenesis of pancreatic cancer.^{17,19} A strong trend was also noted for higher mean serum HSP70 levels in the patients with chronic

pancreatitis compared with normal controls (0.40 ± 0.057 vs 0.043 ng/mL; $P = 0.053$). Similar to other biomarkers, serum HSP70 level was unable to differentiate between the patients with pancreatic cancer and those with chronic pancreatitis. Figure 3 describes the data in a box plot diagram.

Serum HSP70 had a sensitivity of 74% for the detection of pancreatic cancer, which is comparable to the sensitivity of any of the currently available biomarkers (Table 2). Interestingly, this level of sensitivity is much higher than HSP27 (62%), which was recently studied by Liao et. al²¹ for the diagnosis of pancreatic cancer. The specificity and PPV of serum HSP70 for the diagnosis of pancreatic cancer is impressively high at 90% and 95%, respectively. However, a single pair of values for sensitivity and specificity is insufficient to describe the full range of diagnostic performance of a serum tumor marker test. Therefore, ROC curves, which display various sensitivities and false-positive rates at different cutoff levels, were plotted as a better indicator of effectiveness of the diagnostic test. The ROC curve plotted to compare serum HSP70 values between pancreatic cancer and normal controls showed an impressive AUC of 0.794 with 95% CI of 0.67–0.91, which suggests its clinical usefulness as a biomarker. A recent study evaluating combined use of serum biomarkers for detection of pancreatic cancer reported AUC of 0.805, 0.749, and 0.705 for CA 19-9, CA 242, and CEA, respectively.¹³ Area-under-the-curve results on ROC curves in our study are comparable with CA 19-9 and better than that of CA 242 and CEA.

Several studies have evaluated various tumor markers for the diagnosis of pancreatic cancer. Currently available seromarkers, which are used in the diagnosis of pancreatic cancer, are based on detecting circulating mucins, which are secreted into the blood circulation by pancreatic cancer cells.^{25,23} Carcinoembryonic antigen, CA19-9, and CA242 are well-recognized mucins, which are measured in the serum of patients undergoing diagnostic or prognostic evaluation of pancreatic cancer (Table 2).^{21,23–25} Of these, CA 19-9 is currently the most widely used. However, CA 19-9 is a sialylated Lewis blood group antigen and 10% to 47% of patients may be Lewis^{a–b–} genotype and may not express CA 19-9.²⁸ Because of this limitation of CA 19-9, a host of other serum biomarkers have been tested (including M2 pyruvate kinase,²⁹ macrophage inhibitory cytokine 1,¹⁵ insulin like growth factor binding protein,³⁰ and CEA cell adhesion molecule)¹⁴ with varying degrees of success.

It has been suggested that evaluating a combination of biomarkers may be more predictive than assessment of a single marker. A recent multivariate analysis by Pasanen et al²⁷ evaluated 6 serum biomarkers and developed a diagnostic score with a sensitivity of 73% and specificity of 82%. Multiple genetic markers such as *k-Ras*,³¹ p53,³² mucins like MUC1 and 5AC,³² p21,³² SMA-D4,³² BCL2,³² and gene expression microarray³³ are also being investigated as potential biomarkers of pancreatic cancer. However, these genetic markers can only be tested in pancreatic tissue, which is generally not available until surgical intervention.³⁴ Additional emerging markers (iC3b, salivary mRNA, and PAM4) are also being considered in clinical trials (Table 3).^{35–37}

Our data clearly suggest that serum HSP70 has strong potential as an important biomarker for detection of pancreatic cancer, with a sensitivity and specificity of 74% and 90%, respectively. These results are among the highest reported for any biomarker for pancreatic

cancer reported in the literature. Further evaluation of the role of serum HSP70 for surveillance and screening of pancreatic cancer will need to include validation with prospective multicenter clinical trials in patients with pancreatic cancer. Furthermore, comparative studies with CA 19-9 and other biomarkers are also needed. In fact, combining a group of biomarkers including CA 19-9, CA 242, and serum HSP70 may further enhance the sensitivity and specificity of pancreatic cancer detection. Such an approach has been earlier successfully studied using other biomarkers of pancreatic cancer.^{13,27} Additionally, the role of serum HSP70 in intraductal papillary mucinous neoplastic and pancreatic intraepithelial neoplastic lesions needs to be studied further to determine if these neoplastic lesions may be detected at a premalignant stage.

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FIGURE 1.

Scatter diagram depicting individual serum HSP70 levels in 3 groups. Detectable levels of serum HSP 70 were seen in 1 (10%) of 10 patients in the control group, 7 (58%) of 12 patients in the chronic pancreatitis group, and 17 (74%) of 23 patients in pancreatic cancer group.

FIGURE 2.

Box-plot diagram of HSP values. The diagram depicts distribution of HSP70 values (in ng/dL) in 3 groups, mean values being 0.043 in controls, 0.40 in chronic pancreatitis (CP) group and 1.68 in pancreatic cancer (PC) group.

FIGURE 3.

Receiver operating characteristic curves comparing the AUCs for mean serum HSP70 in 3 groups. Pink curve depicts pancreatic cancer versus controls (AUC, 0.7937; 95% CI, 0.67–0.91). Orange curve depicts chronic pancreatitis versus controls (AUC, 0.6933; 95% CI, 0.48–0.90). Red curve depicts pancreatic cancer versus chronic pancreatitis (AUC, 0.681; 95% CI, 0.58–0.77).

TABLE 1

Serum HSP70 Levels

	No. Patients	Serum HSP70 Detected	Serum HSP70 (Mean $\pm$ 1 S.E), ng/dL	P (Compared to Controls)
Group 1: PC	23	17 (74%)	1.68 $\pm$ 0.083	0.013
Group 2: CP	12	7 (58%)	0.40 $\pm$ 0.057	0.053
Group 3: Controls	10	1 (10%)	0.043 $\pm$ 0.0	—

PC indicates Pancreatic cancer; CP, chronic pancreatitis.

TABLE 2

Comparison of Currently Commercially Available Pancreatic Cancer Biomarkers

Biomarker	Year	Author	Sensitivity	Specificity	Comments
Serum CA 242	2003	Ozkan et al ²³	75%	85%	Positive correlation between CA 242 and CA 19-9
Serum CA 19-9	2007	Goonetilleke et al ²⁴	79% (70–90)	82% (68–91)	Meta-analysis, 1990–2005 (22 studies)
Serum CEA	2007	Durraker et al ²⁵	39%	91%	Comparing CEA, CA19-9 and CA 125
Serum HSP27	2009	Liao et al ²¹	62%	95%	Failed to separate PC and CP
Serum HSP70	2011	Dutta et al (this study)	74%	90%	PPV = 95%, ROC comparable to CA 19-9

TABLE 3

Other Emerging Pancreatic Cancer Biomarkers, Not Commercially Available

Biomarker	Year	Author	Sensitivity (%)	Specificity (%)	Comments
Serum M2 pyruvate kinase	2007	Kumar et al ²⁹	72	89	PPV, 58%; NPV, 94%
Ex vivo PAM4	2007	Gold et al ³⁵	82	85	Small sample size (n = 12)
Salivary mRNA (ACRV1)	2009	Wong et al ³⁶	93	85	Limited by small sample size
Serum iC3b	2009	Marten et al ³⁷	77	95	Further increased by combining with CA
Serum REG4	2010	Takayama et al ³⁸	95	64	Better ROC than CA 19-9