Reduced Plasma Level of CXC Chemokine Ligand 7 in Patients with Pancreatic Cancer

Junichi Matsubara¹,², Kazufumi Honda¹, Masaya Ono¹, Yoshinori Tanaka³, Michimoto Kobayashi³, Giman Jung³, Koji Yanagisawa⁴, Tomohiro Sakuma⁴, Shoji Nakamori⁵, Naohiro Sata⁶, Hideo Nagai⁶, Tatsuya Ioka⁷, Takui Okusaka⁸, Tomoo Kosuge⁹, Akihiko Tsuchida⁹, Masashi Shimahara¹⁰, Yohichi Yasunami¹¹, Tsutomu Chiba², Setsuo Hirohashi¹, and Tesshi Yamada¹

Abstract

Background: Early detection is essential to improve the outcome of patients with pancreatic cancer. A noninvasive and cost-effective diagnostic test using plasma/serum biomarkers would facilitate the detection of pancreatic cancer at the early stage.

Methods: Using a novel combination of hollow fiber membrane–based low-molecular-weight protein enrichment and LC-MS-based quantitative shotgun proteomics, we compared the plasma proteome between 24 patients with pancreatic cancer and 21 healthy controls (training cohort). An identified biomarker candidate was then subjected to a large blinded independent validation (n = 237, validation cohort) using a high-density reverse-phase protein microarray.

Results: Among a total of 53,009 MS peaks, we identified a peptide derived from CXC chemokine ligand 7 (CXCL7) that was significantly reduced in pancreatic cancer patients, showing an area under curve (AUC) value of 0.84 and a P value of 0.00005 (Mann–Whitney U test). Reduction of the CXCL7 protein was consistently observed in pancreatic cancer patients including those with stage I and II disease in the validation cohort (P < 0.0001). The plasma level of CXCL7 was independent from that of CA19-9 (Pearson’s r = 0.289), and combination with CXCL7 significantly improved the AUC value of CA19-9 to 0.961 (P = 0.002).

Conclusions: We identified a significant decrease of the plasma CXCL7 level in patients with pancreatic cancer, and combination of CA19-9 with CXCL7 improved the discriminatory power of the former for pancreatic cancer.

Impact: The present findings may provide a new diagnostic option for pancreatic cancer and facilitate early detection of the disease. Cancer Epidemiol Biomarkers Prev; 20(1); 160–71. ©2011 AACR.

Introduction

Pancreatic adenocarcinoma is one of the most aggressive and lethal of diseases. The overall 5-year survival rate of patients with pancreatic cancer is less than 5%, which is the lowest among the more common cancers (1, 2), and the disease is the fifth leading cause of cancer death in Japan and the fourth in the United States, with greater than 23,000 estimated annual deaths in Japan and greater than 33,000 in the United States (3, 4). The 5-year survival rate of patients who were able to undergo surgical resection reaches 20% to 40% (5, 6), but the majority of pancreatic cancer patients have already developed lymph node and/or distant organ metastasis at their first clinical presentation, and only about 20% of patients are able to undergo radical resection (7, 8). The introduction of gemcitabine has significantly improved the overall survival of patients with unresectable pancreatic cancer, but their median survival period still remains about 6 months (9–11). These statistics...
demonstrate that early detection is essential for improving the outcome of patients with pancreatic cancer. Computed tomography (CT), magnetic resonance imaging (MRI), and positron emission tomography (PET) are not cost-effective for the screening of pancreatic cancer because of the relatively low incidence of the disease. If a noninvasive and cost-effective screening test employing plasma/serum markers could be devised, it would significantly facilitate the early detection of pancreatic cancer. However, no biomarker suitable for screening of pancreatic cancer is currently available (12). CA19-9 is an established biomarker useful for the follow-up of pancreatic cancer patients receiving treatment, but has not been recommended for cancer screening because of its insufficient sensitivity and specificity (7, 13). Therefore, the discovery of a new biomarker that would be able to supplement CA19-9 has been anticipated. Recently, advanced proteomic technologies based on mass spectrometry (MS) have been increasingly applied to studies of clinical samples to identify new biomarkers of various diseases (14) including pancreatic cancer (12, 15). It is anticipated that alterations in the protein content of clinical samples reflect the biological status of patients more directly than those in mRNA (16). We previously developed a new shotgun proteome platform, 2-Dimensional Image Converted Analysis of Liquid chromatography and mass spectrometry (2DICAL; ref. 17). 2DICAL is highly advantageous for clinical proteomics because of its high quantification accuracy and throughput. Using 2DICAL, we have been able to identify several plasma/serum biomarkers useful for cancer detection and therapy tailoring (18–20).

The serum/plasma proteome accumulates a large variety of disease-related alterations and is considered to be a rich source of biomarkers. However, for proteomic analysis of blood samples, the efficient depletion of a handful of particularly abundant proteins, such as albumin and immunoglobulin, has been challenging (21). Recently, we developed a novel method for the pretreatment of serum/plasma using the high-performance hollow fiber membrane (HFM) filtration technique (22). This method employs multistage filtration and cascaded cross-flow processes, enabling fully automated separation of proteins below a predetermined molecular weight (22). As the more abundant plasma proteins generally have relatively large molecular weights, they can be efficiently eliminated using the HFM technique.

To identify new biomarkers that might be useful for the early detection of patients with pancreatic cancer, we performed a comprehensive analysis of low-molecular-weight (LMW) plasma proteins in these patients using a combination of the HFM and 2DICAL techniques. A large variety of LMW proteins are known to be secreted from diseased tissues and can serve as good diagnostic biomarkers for various diseases (23, 24). Here, we report the identification and validation of an LMW chemotactic cytokine, CXC chemokine ligand 7 (CXCL7), as a novel biomarker for pancreatic cancer.

Patients and Methods

Plasma samples

Plasma samples were collected prospectively from 282 individuals (K. Honda, T. Okusaka, K. Felix, S. Nakamori, N. Sata, H. Nagai, et al., manuscript submitted) including healthy volunteers and newcomers to mainly departments of gastroenterology between August 2006 and October 2008 at the following 7 hospitals in Japan: National Cancer Center Hospital (NCCH), Osaka National Hospital (ONH), Jichi Medical School Hospital, Osaka Medical College (OMC), Tokyo Medical University Hospital (TMUH), Osaka Medical Center for Cancer and Cardiovascular Diseases, and Fukuoka University Hospital. This multi-institutional collaborative study group was organized by the “Third-Term Comprehensive Control Research for Cancer” conducted by the Ministry of Health, Labour and Welfare of Japan, and as part of the International Cancer Biomarker Consortium (25). The procedures used for collection and storage were kept uniform for all plasma samples.

The 282 plasma samples were split into 2 study sets (referred to as the training and validation cohorts). The training cohort comprised 45 individuals including patients with untreated pancreatic cancer at NCCH (n = 19) and TMUH (n = 5), and healthy controls at NCCH (n = 2), TMUH (n = 9), OMC (n = 6), and ONH (n = 4). The validation cohort comprised 237 individuals including 140 patients with pancreatic cancer, 10 patients with chronic pancreatitis, and 87 healthy controls. All patients diagnosed as having pancreatic cancer had histologically or cytologically proven ductal adenocarcinoma. Demographic and laboratory data are summarized in Table 1. The staging of pancreatic cancer was in accordance with the TNM classification of the International Union against Cancer (UICC).

Blood was collected in a tube with EDTA at the time of diagnosis. The plasma was separated by centrifugation and frozen at –80°C until analysis. Samples showing macroscopic evidence of hemolysis were excluded from the current analysis. Written informed consent was obtained from every subject before blood collection. The protocol of this study was reviewed and approved by the institutional ethics committee boards of each participating institution.

Depletion of high-molecular-weight plasma proteins

The plasma samples of the training cohort were filtered through a 0.22-μm pore size filter. Five hundred microliters of the sample was diluted by adding 3.5 mL of 25 mmol/L of ammonium bicarbonate buffer (pH 8.0). The total 4 mL of the diluted plasma was processed as previously described (22). After 1 hour of fully automated operation, LMW proteins with molecular weights smaller than 60 kDa were recovered (Supplementary Fig. S1) and lyophilized.

The concentration of β2-microglobulin before and after HFM treatment was measured using an ELISA kit (Human Beta-2 Microglobulin ELISA Kit: Alpha Diagnostic Intl. Inc.) to ensure consistent recovery.
Table 1. Clinicopathologic characteristics of individuals in training and validation cohorts

<table>
<thead>
<tr>
<th></th>
<th>Healthy control</th>
<th>Cancer</th>
<th>P</th>
<th>Healthy control</th>
<th>Cancer</th>
<th>P</th>
<th>Chronic pancreatitis</th>
<th>P</th>
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<tr>
<td><strong>No. of patients</strong></td>
<td>21</td>
<td>24</td>
<td>0.205&lt;sup&gt;a&lt;/sup&gt;</td>
<td>87</td>
<td>140</td>
<td>0.485&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10</td>
<td>0.487&lt;sup&gt;a&lt;/sup&gt;</td>
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<td></td>
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<td>8</td>
<td></td>
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<tr>
<td>Female</td>
<td>4</td>
<td>9</td>
<td>&lt;0.001</td>
<td>31</td>
<td>57</td>
<td>2</td>
<td>0</td>
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<td><strong>Age, y, mean (SD)</strong></td>
<td>40 (13)</td>
<td>64 (7)</td>
<td>&lt;0.001</td>
<td>43 (16)</td>
<td>66 (10)</td>
<td>&lt;0.001</td>
<td>58 (13)</td>
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<td>Body or tail</td>
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<td></td>
<td>–</td>
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<td>I</td>
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<td>70</td>
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<td><strong>CA19-9 median, U/mL</strong></td>
<td>5.5</td>
<td>1,109</td>
<td>&lt;0.001</td>
<td>10.2</td>
<td>476</td>
<td>&lt;0.001</td>
<td>5.2</td>
<td>0.06</td>
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<td>19</td>
<td></td>
<td>4</td>
<td>110</td>
<td></td>
<td>0</td>
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<td><strong>DUPAN-2 median, U/mL</strong></td>
<td>12</td>
<td>540</td>
<td>&lt;0.001</td>
<td>12</td>
<td>375</td>
<td>&lt;0.001</td>
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<td>19</td>
<td></td>
<td>0</td>
<td>92</td>
<td></td>
<td>1</td>
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<td><strong>CEA median, ng/mL</strong></td>
<td>1.7</td>
<td>6.0</td>
<td>&lt;0.001</td>
<td>1.7</td>
<td>3.5</td>
<td>&lt;0.001</td>
<td>2.6</td>
<td>0.110</td>
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<td>&gt;5.0 (ULN), no. of patients</td>
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<td></td>
<td>5</td>
<td>49</td>
<td></td>
<td>1</td>
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<tr>
<td><strong>Total bilirubin median, mg/dL</strong></td>
<td>0.5</td>
<td>0.4</td>
<td>0.688</td>
<td>0.5</td>
<td>0.5</td>
<td>0.574</td>
<td>0.5</td>
<td>0.4</td>
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<td>&gt;1.2 (ULN), no. of patients</td>
<td>0</td>
<td>2</td>
<td></td>
<td>4</td>
<td>18</td>
<td></td>
<td>0</td>
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<tr>
<td><strong>CXCL7</strong></td>
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<td></td>
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<tr>
<td>Mass spectrometry peak intensity&lt;sup&gt;b&lt;/sup&gt;, mean (SD)</td>
<td>332 (240)</td>
<td>138 (346)</td>
<td>&lt;0.001&lt;sup&gt;d&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Protein intensity&lt;sup&gt;c&lt;/sup&gt;, mean (SD)</td>
<td>4.14 (0.18)</td>
<td>3.83 (0.28)</td>
<td>0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.18 (0.14)</td>
<td>3.92 (0.28)</td>
<td>0.001&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3.99 (0.10)</td>
<td>&lt;0.001&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

NOTE. Wilcoxon test was applied to assess differences in values.
Abbreviations: CEA, carcinoembryonic antigen; NA, not applicable; ULN, upper limit of normal.
<sup>a</sup>Calculated by Fisher’s exact test.
<sup>b</sup>Intensity of the corresponding peak measured by quantitative mass spectrometry.
<sup>c</sup>Measured using reverse-phase protein microarrays (logarithmic variable).
<sup>d</sup>Calculated by Mann-Whitney U-test.
<sup>e</sup>Calculated by Welch’s t-test.
<sup>f</sup>Compared with healthy controls.
Liquid chromatography/mass spectrometry

The HFM-treated samples were digested with sequencing grade-modified trypsin (Promega) and analyzed in duplicate using a nano-flow high-performance liquid chromatography (HPLC; NanoFrontier nLC, Hitachi High-technologies) connected to an electrospray ionization quadrupole time-of-flight (ESI-Q-TOF) mass spectrometer (Q-Tof Ultima, Waters).

MS peaks were detected, normalized, and quantified using the in-house ZDICAL software package, as described previously (17). A serial identification (ID) number was applied to each of the MS peaks detected (1 to 53,009). The stability of LC-MS was monitored by calculating the correlation coefficient (CC) and coefficient of variance (CV) of every measurement. The mean CC ± SD and CV ± SD for all 53,009 peaks observed in the 45 duplicate runs were as high as 0.946 ± 0.042 and as low as 0.053 ± 0.010, respectively.

Protein identification by tandem MS (MS/MS)

Peak lists were generated using the Mass Navigator software package (version 1.2; Mitsui Knowledge Industry) and searched against the SwissProt database (downloaded on April 22, 2009) using the Mascot software package (version 2.2.1; Matrix Science). The search parameters used were as follows. A database of human proteins was selected. Trypsin was designated as the enzyme, and up to 1 missed cleavage was allowed. Mass tolerances for precursor and fragment ions were 0.05 Da and 0.2 Da, respectively. The score threshold was set to $P < 0.05$ based on the size of the database used in the search. If a peptide was matched to multiple proteins, the protein name with the highest Mascot score was selected.

Western blot analysis

Primary antibodies used were a rabbit polyclonal antibody against platelet basic protein (PBP) precursor (Sigma) and a mouse monoclonal antibody against human complement C3b-α (PROGEN). The anti-PBP antibody recognizes all the known cleaved forms of PBP including CTAP-III and NAP-2. Six microliters of 1:10 diluted plasma sample was separated by SDS-PAGE and electroblotted onto a polyvinylidene difluoride (PVDF) membrane. The membrane was then incubated with the primary antibody and subsequently with the relevant horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse IgG, as described previously (26, 27). Blots were developed using an enhanced chemiluminescence (ECL) detection system (GE Healthcare).

Reverse-phase protein microarray

The plasma samples were passed through IgY microbeads (Seppro-IgY12, Sigma-Aldrich) using an automated Magtration System SA-1 (Precision System Science) in accordance with the manufacturer’s instructions to reduce the 12 most abundant plasma proteins. The flow-through portion was serially diluted 1:50, 1:100, 1:200, and 1:400 using a Biomek 2000 Laboratory Automation Robot (Beckman Coulter) and randomly plotted onto ProteoChip glass slides (Proteogen) in quadruplicate in a 6,144-spot/slide format using a Protein Microarrayer Robot (Kaken Genexs). The spotted slides were incubated overnight with the anti-PBP precursor antibody and then with biotinylated anti-rabbit IgG (Vector Laboratories) and subsequently with streptavidin-HRP conjugate (GE Healthcare). The peroxidase activity was detected using the Tyramide Signal Amplification (TSA) Cyanine 5 System (PerkinElmer). The slides were counterstained with Alexa Fluor 546-labeled goat anti-human IgG (Invitrogen; spotting control).

The stained slides were scanned on a microarray scanner (InnoScan 700AL; Innopsys). Fluorescence intensity, determined as the mean value of quadruplicate samples, was determined using the Mapix software package (Innopsys). All determined intensity values were transformed into logarithmic variables.

The reproducibility of the reverse-phase protein microarray assay was determined by repeating the same experiment, as reported previously (28). A plasma sample after reduction of the 12 most abundant plasma proteins was serially diluted within a range of 25- to 6,400-fold. Each diluted sample was spotted in quadruplicate onto glass slides and blotted with the anti-PBP antibody. In a representative quality control experiment, the CC value was 0.980 between days and the median CV was 0.047 among the quadruplicates.

Multiplex assay

The levels of CXCL7 in plasma samples were measured using a Milliplex Human Cytokine/Chemokine panel III kit (Millipore) in accordance with the manufacturer’s instructions.

Statistical analysis

Statistical significance of intergroup differences was assessed with the Wilcoxon test, Mann–Whitney U test, Welch’s $t$ test, or Fisher’s exact test, as appropriate. The area under the curve (AUC) of the receiver-operating characteristic (ROC) was calculated for each marker to evaluate its diagnostic significance. A composite index of 2 markers was generated using the results of multivariate logistic regression analysis, which also enabled the calculation of sensitivity, specificity, and the ROC curve. Statistical analyses were performed using an open-source statistical language R (version 2.7.0) with the optional module Design package.

Results

Plasma proteins associated with pancreatic cancer

A plasma sample from 1 healthy volunteer was processed 3 times using the HFM filtration technique. The concentration of β2-microglobulin before and after HFM treatment was measured. The recovery rates were...
25.11%, 25.73%, and 29.16%, respectively. Although the rates were seemingly low, the HFM treatment was highly reproducible with a CV of 0.081 and the amount of β2-microglobulin relative to total protein was increased 150 to 200-fold after HFM treatment.

To identify a diagnostic biomarker for pancreatic cancer, we compared the plasma LMW proteome between 24 patients with pancreatic cancer and 21 healthy controls (training cohort) using 2DICAL. Among a total of 53,009 independent MS peaks detected within the range 250 to 1,600 m/z and within a time range of 20 to 70 minutes, we found that 140 peaks had discriminatory ability with an AUC of above 0.800. Figure 1A is a representative 2-dimensional view of all the MS peaks displayed with m/z along the x-axis and the retention time (RT) of LC along the y-axis. Twenty-five MS/MS spectra acquired from those 140 peaks were recurrently matched to 10 proteins in the database with a Mascot score of greater than 30 (Supplementary Table S1). Notably, one MS peak (ID 54) matched the amino acid sequence of the CXCL7 gene product (Swiss-Prot_P02775) with the highest score of 99.6 (Supplementary Fig. S2). Figure 1B shows the CXCL7-derived MS peak (ID 54, at 863 m/z and 50.2 minutes) that appeared in a representative pancreatic cancer patient and a healthy individual. Figure 1C demonstrates the distribution of the MS peak (ID 54) in patients with pancreatic cancer (red) and healthy controls (blue) in the training cohort (AUC = 0.839; P = 4.54 x 10^{-5}; Mann–Whitney U test). The differential expression and identification of CXCL7 was confirmed by immunoblotting (Fig. 1D).
Validation of reduced CXCL7 in pancreatic cancer patients

The level of plasma CXCL7 was quantified in 12 patients with pancreatic cancer and 12 healthy individuals in the training cohort using multiplex assay. Consistent with 2DICAL, CXCL7 was found to be significantly decreased in patients with pancreatic cancer (mean ± SD, 744 ± 182 ng/mL) in comparison with healthy controls (1,355 ± 386 ng/mL; \( P = 0.0003 \)). To further verify and validate the reduction of plasma CXCL7 in pancreatic cancer patients, 280 plasma samples [including 43 samples from the training cohort and new 237 samples (validation cohort)] were randomly plotted into a reverse-phase protein microarray and blotted with anti-PBP antibody (Fig. 2). Two samples from healthy controls in the training cohort were excluded due to an insufficient sample volume. Quadruplicate spots for representative cases and controls with high and low levels of CXCL7 are shown in the right panels of Figure 2.

The results of reverse-phase protein microarray were well correlated with those of multiplex assay (Pearson’s \( r = 0.65; P = 0.0006 \); Supplementary Fig. S3). Microarray analysis also showed a significant reduction of the plasma CXCL7 level in the pancreatic cancer patients of the training cohort (\( P = 5.96 \times 10^{-5} \); Welch’s \( t \) test; Fig. 3A and Table 1) with an AUC value of 0.872 (95% CI: 0.865–0.994). The reduction of plasma CXCL7 was further validated in a larger independent cohort (validation cohort; \( P = 1.40 \times 10^{-16} \); Fig. 3C and AUC value of 0.859, 95% CI: 0.792–0.959; Fig. 3B). Because there was a difference in age distribution between the cancer patients and healthy controls of the validation cohort (Table 1), we performed a subgroup analysis of 79 pancreatic cancer patients (median age, 61) and 20 healthy controls (median age, 60) aged 50 to 70 years. The reduction of plasma CXCL7 in patients with pancreatic cancer was statistically significant even in this subgroup (\( P = 0.0001 \)), indicating that the decrease of the CXCL7 level was not merely due to the difference of age distribution between the pancreatic cancer patients and controls.

CXCL7 was significantly reduced in patients with any stage of pancreatic cancer (Table 2), including those with stage I (<0.001) and II (<0.001) disease. The significant alteration evident in early-stage patients indicated that the reduction of plasma CXCL7 is an early event in pancreatic carcinogenesis and may precede the development of cancer. The persistent presence of inflammation is known to promote carcinogenesis in various organs, and chronic pancreatitis is suspected to be one a precancerous condition for pancreatic cancer, although opinions on this issue vary. We measured the plasma level of CXCL7 in a small number of patients diagnosed as having chronic pancreatitis (\( n = 10 \)) using the reverse-phase protein microarray (Table 1). The CXCL7 levels in patients with chronic pancreatitis were significantly lower than those in healthy controls (\( P = 0.0002 \)), but slightly higher than those in patients with pancreatic cancer (\( P = 0.095 \); Fig. 3C).

Complementation of CA19-9 by CXCL7

CA19-9 is an established biomarker that has long been used for the diagnosis of pancreatic cancer. We found that the levels of CXCL7 and CA19-9 were not mutually correlated (Pearson’s \( r = 0.289 \)) and that combination with CXCL7 significantly improved the ability of CA19-9 to distinguish patients with pancreatic cancer from healthy controls: the AUC value improved to 0.965 (95% CI: 0.865–0.994) in the training cohort (\( P = 0.026 \)) and to 0.961 (0.932–0.979) in the validation cohort (\( P = 0.002 \); Fig. 3D). The AUC values of CA19-9 in the 2 cohorts (Fig. 3D) were comparable with those reported previously (29–31).

Even among individuals with normal levels of CA19-9 (<37 U/mL; a cutoff value widely used in clinical practice), CXCL7 was significantly reduced in pancreatic cancer patients in both the training [\( P = 0.014 \) and AUC = 0.853 (95% CI: 0.650–0.957; Fig. 4A and B)] and validation [\( P < 0.0001 \) and AUC = 0.834 (95% CI: 0.747–0.899; Fig. 4B and C)].

Because of the low prevalence of pancreatic cancer, any screening biomarker must have high specificity (32). The sensitivity/specificity of CA19-9 (cutoff: 37 U/mL) were 79%/89% in the training cohort and 79%/95% in the validation cohort, consistent with previous reports (32). If we defined the cutoff for CXCL7 as a level at which 95% of healthy individuals would be excluded, 83% of pancreatic cancer patients in the training cohort and 84% in the validation cohort would be detected using the combination of CXCL7 and CA19-9 (Supplementary Table S2).

Discussion

Early detection and subsequent radical surgical resection would most likely provide a chance of cure for patients with pancreatic cancer (7). However, patients with early-stage pancreatic cancer are generally asymptomatic and have little opportunity to undergo imaging and/or other diagnostic procedures until their disease becomes advanced. If a sensitive, but minimally invasive and cost-effective, plasma/serum test were available, it would be effective for alerting patients with early pancreatic cancer and offer them a chance to receive prompt and effective medical attention. In the present study, we compared the plasma LMW proteome between patients with pancreatic cancer and healthy controls using a new proteome platform, 2DICAL (Fig. 1), and found a significant decrease of the plasma CXCL7 level in patients with pancreatic cancer (Fig. 1B and C). The result of quantitative LC-MS was then verified using 3 different methods: immunoblotting (Fig. 1D), multiplex, and reverse-phase protein microarray (Figs. 2 and 3) assays. We further validated the significant decrease of CXCL7 in a larger independent cohort (validation cohort). The level of plasma CXCL7 was confirmed to be decreased reproducibly in patients with pancreatic cancer including those with Stage I and II disease (Table 2). CXCL7 did not...
surpass the sensitivity of CA19-9, but was able to supplement it. Combination with CXCL7 significantly improved the sensitivity of CA19-9 (Fig. 3D and Supplementary Table S2).

In addition to 2DICAL, we utilized 2 state-of-the-art proteome technologies. The proteome analysis of plasma/serum samples has been hampered by the prominence of a handful of abundant proteins such as...
albumin and immunoglobulin. It is anticipated that the remaining proteins contain an unexplored archive of disease-driven information, but account for only about 1% of the entire human plasma proteome (24). To reduce the complexity of the plasma proteome, we used HFM filtration technology. Our HFM devise can separate and concentrate LMW plasma proteins in a fully automated manner (22) and allows identification of any biomarker candidate that is present at a level of 1 and g/mL. This discovery justifies the future application of the HFM system to more detailed proteome studies aimed at plasma/serum biomarker discovery. The other technology we employed is high-density reverse-phase protein microarray. The protein content of any human sample varies according to the individual, and therefore it is essential to distinguish biomarker candidates from simple interindividual heterogeneity. However, such distinction is possible only by comparing a statistically sufficient number of cases and controls. Our high-density protein microarrays require a minimal sample volume of the nanoliter order and make it possible to measure the quantity of any candidate biomarker protein in a statistically sufficient number of cases and controls (>300 samples; ref. 28) for judgment of its clinical potential in a single experiment.

LMW chemotactic cytokines have been implicated in various biological processes, such as leukocyte migration, angiogenesis, hematopoiesis, atherosclerosis, and cancer migration and metastasis. CXCL7, also known as PBP, is one of the members of the angiogenic ELR+ CXC chemokine family (33). It is reportedly produced and stored in platelets, monocytes, neutrophils, and megakaryocytes. Secreted CXCL7 binds to CXC chemokine receptor 2 (CXCRII) on endothelium and mediates angiogenesis through activation of the Ras/Raf/mitogen-activated protein kinase (MAPK) and PI3K/AKT/mTOR signaling pathways (33, 34). The histology of pancreatic ductal adenocarcinoma is often characterized by hypovascularization. The reduction of circulating CXCL7 in patients with pancreatic cancer may play a certain role in the suppression of angiogenesis.

Recently, reduction in the level of serum CXCL7 has been reported to be a biomarker for advanced myelodysplastic syndrome (35). In contrast, CXCL7 is increased in the pulmonary venous blood of lung cancer patients and is significantly decreased after curative surgical resection of the lung lesions. Of particular interest is the fact that the increment of CXCL7 is detectable several months before diagnosis of lung cancer (36). We observed a reduction of CXCL7 in 10 patients with chronic pancreatitis; but, examination of a larger number of patients will be needed before any definite conclusion can be reached.

CXCL7 is N-terminally truncated by cathepsin G-like enzymes and converted to other types of chemokines with distinct functions such as connective tissue-activating peptide III (CTAP-III) and neutrophil-activating peptide 2 (NAP-2; refs. 37, 38). One possible explanation for the reduction of plasma CXCL7 in patients with pancreatic cancer is degradation by certain exoproteases (39). Matrix metalloproteinase-9 (MMP9) has been reported to degrade CXC chemokines (40). MMP9 is often upregulated in pancreatic cancer cells and secreted into plasma (41). However, in this study, the precise molecular mechanisms behind the reduction of plasma CXCL7 in patients with pancreatic cancer remained unexplained.

Because the process of pancreatic carcinogenesis is probably mediated by various molecular pathways (42), the diagnosis of pancreatic cancer using a single biomarker may not be realistic, and a combination of different biomarkers with distinct spectra would appear to be a more realistic alternative. CA19-9 is the most widely used serum biomarker for pancreatic cancer; but, its sensitivity and specificity have been recognized

Table 2. Plasma CXCL7 level according to clinical stage of pancreatic cancer

<table>
<thead>
<tr>
<th></th>
<th>Pancreatic cancer patients</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stage I</td>
<td>Stage II</td>
</tr>
<tr>
<td>Training cohort</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of cases</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>CXCL7, mean (SD)</td>
<td>3.67 (-)</td>
<td>3.93 (0.24)</td>
</tr>
<tr>
<td>P (vs. healthy controls)</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Validation cohort</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of cases</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>CXCL7, mean (SD)</td>
<td>3.89 (0.34)</td>
<td>3.96 (0.25)</td>
</tr>
<tr>
<td>P (vs. healthy controls)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

NOTE. Welch’s t test was applied to assess differences in values.

* Measured using reverse-phase protein microarrays.

b Two patients whose samples were not available for reverse-phase protein microarrays were excluded.
to be unsatisfactory for pancreatic cancer screening (7, 12). We demonstrated that CXCL7 significantly improved the discriminatory ability of CA19-9, and this improvement was reproducibly validated in a large multi-institutional cohort. However, further independent validation by other investigators is still mandatory before its clinical application can be warranted (15, 29–31, 43).

Figure 3. A and C, plasma levels (in arbitrary units) of CXCL7 in healthy controls, patients with pancreatic cancer, and patients with chronic pancreatitis in the training (A) and validation (C) cohorts. Horizontal lines represent the average levels of CXCL7. B, ROC analyses for the discriminatory value of CXCL7 in the training (dotted line) and validation (solid line) cohorts. D, ROC analyses for the discriminatory value of CA19-9 and the composite index of CA19-9 and CXCL7 in the training and validation cohorts.
The primary goal of the present study was to discover new biomarkers useful for the early detection of pancreatic cancer in an asymptomatic population. Aberrations of circulating CXCL7 have also been reported in other premalignant conditions. The present study has not only explored the utility of CXCL7 as a biomarker, but also provided a novel insight into the chemokine-mediated reactions that occur during early carcinogenesis.

**Disclosure of Potential Conflicts of Interest**

These sponsors had no role in the design of the study, the collection of the data, the analysis and interpretation of the data, the decision to submit the manuscript for publication, or the writing of the manuscript.

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