Identification of Serum Biomarker Signatures Associated with Pancreatic Cancer

Christer Wingren1,2, Anna Sandström1,2, Ralf Segersvärd4, Anders Carlsson1,2, Roland Andersson3, Matthias Lohr4, and Carl A. K. Borrebaeck1,2

Abstract

Pancreatic cancer is an aggressive disease with poor prognosis, due, in part, to the lack of disease-specific biomarkers that could afford early and accurate diagnosis. With a recombinant antibody microarray platform, targeting mainly immunoregulatory proteins, we screened sera from 148 patients with pancreatic cancer, chronic pancreatitis, autoimmune pancreatitis (AIP), and healthy controls (N). Serum biomarker signatures were derived from training cohorts and the predictive power was evaluated using independent test cohorts. The results identified serum portraits distinguishing pancreatic cancer from N [receiver operating characteristics area under the curve (AUC) of 0.95], chronic pancreatitis (0.86), and AIP (0.99). Importantly, a 25-serum biomarker signature discriminating pancreatic cancer from the combined group of N, chronic pancreatitis, and AIP was determined. This signature exhibited a high diagnostic potential (AUC of 0.88). In summary, we present the first prevalidated, multiplexed serum biomarker signature for diagnosis of pancreatic cancer that may improve diagnosis and prevention in premalignant diseases and in screening of high-risk individuals. Cancer Res; 72(10); 2481–90. © 2012 AACR.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is characterized by a rapid tumor progression, early metastasis, and unresponsiveness to most conventional chemotherapies (1, 2). With an overall 5-year survival rate of less than 2% to 3%, it is one of the most lethal types of malignancies (3, 4). Even though PDAC only represents the tenth most common form of cancer, it is the fourth leading cause of cancer-related death in the Western world (3–5).

Today, surgery remains the only potentially curative therapy for PDACs. However, because of lack of early and disease-specific clinical symptoms or signs in a majority of patients with PDACs, the disease is usually in an incurable stage at the time of diagnosis, either with distant metastases or with a locally advanced, and thus irresectable tumor (3–5). The remaining 10% to 15% of patients have their tumors confined to the pancreas and have reported median survival of about 20 months after resection and adjuvant chemotherapy (6). However, significantly better outcomes have been reported for smaller tumors detected at an earlier stage. A 5-year survival rate of 30% to 60% in tumors less than 20 mm in size and even exceeding 75% in tumors less than 10 mm have been reported (3–5). Hence, earlier diagnosis could potentially improve diagnosis.

A variety of noninvasive methodologies, including endoscopic ultrasound, computed tomography, and/or endoscopic retrograde cholangiopancreatography, are used for PDAC diagnosis (2, 3, 7). Albeit powerful, these imaging methods are not specific for PDAC and not designed for early detection when the tumor is still small and potentially curable, or the early premalignant lesions, such as pancreatic intraepithelial neoplasia (8). Furthermore, they are also unable to tell whether a premalignant mucinous cystic lesion has undergone malignant transformation or to distinguish PDAC from tumor-forming lesions caused by benign conditions such as chronic and autoimmune pancreatitis (3). Hence, PDAC-specific biomarkers would be of great clinical value.

Despite major efforts, molecular fingerprints associated with PDACs from, in particular, crude, nonfractionated serum and plasma remain a challenge (3, 9–12). Among the number of mainly single biomarkers that have been outlined so far, including, for example, C-reactive protein (CRP), CA 242, GDF-15, haptoglobin, M2-pyruvate kinase, serum amyloid A, platelet factor 4, and IGF-binding protein (IGFBP)-1, none have proven to be clinically superior to CA 19-9, the most used biomarker today (3, 10, 11, 13). However, the use of CA 19-9 is significantly hampered by the fact that it has been found to be elevated in both nonmalignant conditions (e.g., pancreatitis and acute cholangitis) and other gastrointestinal cancers (e.g., gastric cancer and colorectal cancer), and thus not PDAC-specific (3, 10, 11, 13). Moreover, CA 19-9 may be absent in about 10% of the population, as subjects who are genotypically...
Lewis a-b- cannot produce the CA 19-9 epitope (3, 10, 11, 13). When screening for PDAC, CA 19-9 has thus only yielded a moderate sensitivity (ranging from 69% to 98%) and specificity (46%–98%; refs. 3, 11, 13, 14).

Motivated by a recent study, where we indicated that affinity proteomics (15, 16) could be used to pinpoint candidate PDAC serum biomarker signatures (17), the current study was undertaken to further analyze the serum proteome of pancreatic diseases. In the present study, we have for the first time prevalidated a serum biomarker signature for PDAC diagnosis, discriminating it from inflammatory states of the pancreas. This diagnostic information could be extracted from crude blood samples, displaying high specificity and sensitivity, by recombinant antibody microarrays interfaced with stringent microarray data analysis procedures. In the future, this could provide novel opportunities for improved diagnosis of PDAC, screening of high-risk groups, such as patients with chronic pancreatitis, cystic lesions of the pancreas, and individuals with cancer inheritance, as well as shedding further light on the underlying and intricate disease biology.

Material and Methods

Serum samples

After informed consent, serum samples were collected at the time of diagnosis, that is, before commencing any therapy, from 2 independent patient cohorts and stored at −80°C. PDAC was verified by histology. Patient cohort 1 was composed of serum samples from 103 patients, diagnosed with PDAC (stage III or IV; n = 34), chronic pancreatitis (n = 16), autoimmune pancreatitis (AIP; n = 23), or healthy individuals (controls, N; n = 30, no clinical symptoms). The patient demographics are described in Table 1. This cohort was also randomly split and used as a training set and test set. Patient cohort 2 was composed of 45 new patients, diagnosed with PDAC (n = 25) or N (controls, n = 20; for patient demographics, see ref. 17), as recently described (17). Patient cohorts 1 and 2 were from clinically independent sets of patients collected at different sites. There was a skewed age distribution between the patients with PDAC and healthy individuals (Table 1), as the patients with cancer were, on average, older than the normal individuals. Consequently, if age was a confounding factor, the older normal samples would have been classified as cancer, and the younger cancer samples would have been classified as normal. However, this was not the case. In fact, using the support vector machine (SVM), all the older normal samples were correctly classified, whereas only 2 of the youngest cancer samples were misclassified. Furthermore, age was not a confounding factor in our previous antibody array profiling studies (e.g., see ref. 17; unpublished observation). A power analysis (see below) was conducted to confirm that the size of the sample cohorts was sufficient to provide a statistical power of more than 80%. The main experiments were carried out on patient cohort 1, whereas cohort 2 was used as an independent data set for validation in one of the experiments (See Supplementary Fig. S1). The serum level of CA 19-9 was measured with a commercially available CA 19-9 ELISA (Abnova).

Antibody microarray analysis

The recombinant antibody microarray analysis was conducted using previously in-house optimized protocols (refs. 15–18; see Supplementary Material and Methods for details). Briefly, 121 human recombinant single-chain Fv (scFv) antibodies, targeting 57 mainly immunoregulatory biomolecules, were used as probes. The specificity, affinity (nanomolar range), and on-chip functionality of the phage-display-derived scFvs (19) was ensured by using (i) stringent selection protocols (19), (ii) multiple clones (≤4) per target molecule, and (iii) an scFv library microarray adapted by molecular design (20, 21). The planar antibody microarrays (array size: 160 × 8, <2 cm2) were prepared by dispensing the antibodies and controls one-by-one (330 pL/drop) with a noncontact spotter. The biotinylated serum samples were screened separately, and specifically bound molecules were visualized by adding fluorescently labeled streptavidin and using a confocal fluorescence scanner. Each individual array data point represents the mean value of 4 replicates. Chip-to-chip normalization was conducted by using a semiglobal normalization approach. In accordance with previous studies (15, 18, 22), the correlation coefficient for spot-to-spot reproducibility and array-to-array reproducibility was 0.99 and 0.94, respectively. Selected antibody specificities and microarray data were validated (Table 2), with a 234 human protein array and a 10-plex cytokine sandwich antibody microarray (MSD), respectively. In addition, several antibody specificities have previously been validated by well-characterized, standardized serum samples, and independent methods, such as mass spectrometry, ELISA, multiplexed...

<table>
<thead>
<tr>
<th>Class</th>
<th>No. of patients</th>
<th>Gender (M/F/unknown)</th>
<th>Mean (SD)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDAC</td>
<td>34</td>
<td>18/12/4</td>
<td>65.0 (10.4)</td>
<td>42–93</td>
</tr>
<tr>
<td>N</td>
<td>30</td>
<td>15/15/0</td>
<td>33.2 (8.6)</td>
<td>24–53</td>
</tr>
<tr>
<td>Chronic pancreatitis</td>
<td>16</td>
<td>12/4/0</td>
<td>48.8 (14.2)</td>
<td>32–73</td>
</tr>
<tr>
<td>AIP</td>
<td>23</td>
<td>11/11/1</td>
<td>42.4 (18.3)</td>
<td>14–74</td>
</tr>
<tr>
<td>All</td>
<td>103</td>
<td>56/42/5</td>
<td>48.2 (18.1)</td>
<td>14–93</td>
</tr>
</tbody>
</table>
Table 2. Summary of serum biomarkers analyzed by the antibody microarrays

<table>
<thead>
<tr>
<th>Antigen (number of clones)</th>
<th>Antigen (number of clones)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiomotin (2)</td>
<td>IL-8 (3)&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>β-galactosidase (1)</td>
<td>IL-9 (3)</td>
</tr>
<tr>
<td>Bruton tyrosine kinase BTK (1)</td>
<td>IL-10 (3)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C1 esterase inhibitor (1)</td>
<td>IL-11 (3)</td>
</tr>
<tr>
<td>C1q (1)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>IL-12 (4)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C1s (1)</td>
<td>IL-13 (2)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C3 (2)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>IL-16 (2)</td>
</tr>
<tr>
<td>C4 (1)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>IL-18 (3)</td>
</tr>
<tr>
<td>C5 (2)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Integrin α-10 (1)</td>
</tr>
<tr>
<td>CD40 (4)</td>
<td>Integrin α-11 (1)</td>
</tr>
<tr>
<td>CD40 ligand (1)</td>
<td>Leptin (1)</td>
</tr>
<tr>
<td>Cholera toxin subunit B (control) (1)</td>
<td>Lewis X (2)</td>
</tr>
<tr>
<td>Digoxin (control) (1)</td>
<td>Lewis Y (1)</td>
</tr>
<tr>
<td>Eotaxin (3)</td>
<td>MCP-1 (3)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Factor B (1)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>MCP-3 (1)</td>
</tr>
<tr>
<td>GLP-1 (1)</td>
<td>MCP-4 (2)</td>
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<td>GLP-1-R (1)</td>
<td>Mucine-1 (6)</td>
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<td>GM-CSF (3)</td>
<td>Procathepsin W (1)</td>
</tr>
<tr>
<td>IFN-γ (2)</td>
<td>Properdin (1)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IgM (1)</td>
<td>PSA (1)</td>
</tr>
<tr>
<td>IL-1α (3)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Rantes (2)</td>
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<tr>
<td>IL-1β (3)</td>
<td>Sialyl Lewis X (1)</td>
</tr>
<tr>
<td>IL-1ra (3)</td>
<td>TGF-β1 (3)</td>
</tr>
<tr>
<td>IL-2 (3)</td>
<td>Tm peptide (1)</td>
</tr>
<tr>
<td>IL-3 (3)</td>
<td>TNF-α (2)</td>
</tr>
<tr>
<td>IL-4 (4)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>TNF-β (4)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL-5 (3)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Tyrosine-protein kinase JAK3 (1)</td>
</tr>
<tr>
<td>IL-6 (4)&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>VEGF (4)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL-7 (2)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Antibody specificity determined by protein array and MSD.
<sup>b</sup>Antibody specificity previously validated by ELISA, MSD, protein array, blocking/spiking experiments, and/or mass spectrometry.

cytokine sandwich antibody microarrays (MSD), and cytometric bead assay, as well as by spiking and blocking experiments (Table 2).

Microarray data analysis

The data analysis was conducted in R (see Supplementary Material and Methods for details). Briefly, a SVM-derived prediction algorithm was used to classify the samples as belonging to 1 of 2 defined groups (e.g., cancer or healthy), using a linear kernel with the cost of constraints set to 1. No attempts were made to tune it to avoid the risk of overfitting. The SVM was trained and tested with a leave-one-out (LOO) cross-validation procedure. In 2 of the comparisons, the training part included the creation of a subpanel of antibodies by selecting the combination of antibodies that displayed the highest discriminatory power in the training set. This selection of antibodies was made, with either a direct or a cross-validated backward elimination strategy. With this approach, condensed candidate biomarker signatures were identified, and these were subsequently validated, using an independent patient cohort. The overall microarray strategy is schematically outlined in Supplementary Fig. S1.

Sensitivity and specificity values were calculated from the SVM decision values using a threshold level of zero. A receiver operating characteristics (ROC) curve was constructed using the SVM decision values for the entire range of sensitivity versus specificity thresholds. The area under the ROC curve (AUC) was calculated and used as a measure of prediction performance. Further, the Wilcoxon P value and the fold change were calculated for each antibody. A power analysis was also conducted to evaluate the sample sizes. The candidate biomarker signatures were reported following the recommendations for tumor marker prognostic studies (23).

Results

Classification of PDAC versus healthy controls (N)

To identify serum biomarker signature associated with PDACs, we conducted differential serum protein expression profiling of PDACs (n = 34) versus N (n = 30). A representative image of an antibody microarray is shown in Fig. 1A, illustrating that dynamic signal intensities, adequate spot morphology, and low nonspecific background binding were obtained. The results showed that 33 nonredundant protein molecules, including for example, both T-helper (TH)1 and TH2 cytokines, were found to be differentially expressed (P < 0.05). All, but the complement proteins C1q and properdin, were upregulated in PDACs (Fig. 1B).

To investigate whether PDACs and N could be distinguished, we ran an SVM LOO cross-validation, based on all antibodies, that is, using unfiltered data. The data showed that the patient cohorts could be classified with a ROC AUC value of 0.94 (Fig. 1C). In Fig. 1D, the samples are plotted by decreasing SVM scores. The candidate biomarker signatures were reported following the recommendations for tumor marker prognostic studies (23).

Prevalidation of condensed biomarker signature for PDAC versus N classification

To test the strength of the classification (based on 64 patients), we first condensed the total number of molecules...
down to the 18 nonredundant biomarkers contributing the most to the classification by combining a LOO procedure with an iterative backward elimination strategy. In this process, the Kullback–Leibler divergence error was minimized and used as a guide for stepwise removal of the antibodies. After each round, the SVM decision values were collected and the corresponding ROC curve and AUC value were calculated. In Fig. 2A, the AUC value is plotted against the number of remaining antibodies, indicating a high and stable classification even when only a few antibodies were included. The 18-analyte serum biomarker signature was composed of a variety of molecules, such as cytokines, complement proteins, and enzymes (Fig. 2B). Next, we applied this 18-analyte signature on a new independent patient group, that is, patient cohort 2 (n = 45; Fig. 2C). The results showed that this biomarker signature allowed stratification of patients into PDAC versus N with a ROC AUC value of 0.95 (Fig. 2D), corresponding to a sensitivity of 88% and specificity of 85%. Hence, these data outlined the first pre-validated serum biomarker signature for an accurate PDAC diagnosis.

**Biomarker signatures differentiating PDAC versus pancreatitis**

To test whether cancer could be differentiated from benign conditions in the pancreas, we compared the serum protein expression profile of PDAC (n = 34) with that of chronic pancreatitis (n = 16) or AIP (n = 23). In the case of PDAC versus chronic pancreatitis, 15 differentially expressed (P < 0.05) serum molecules were identified, of which all but 2 (IL-4 and IL-12) were upregulated in PDAC (Fig. 3A). On the basis of unfiltered data, that is, all antibodies, the results showed that PDACs and chronic pancreatitis could be differentiated with an ROC AUC value of 0.86 (Fig. 3B), corresponding to 97% sensitivity and 69% specificity. A total of 49 serum molecules were found to be differentially expressed in PDAC versus AIP, with all except for C1q and properdin, being upregulated in PDACs (Fig. 3A). Furthermore, again based on unfiltered data, the results showed that PDAC versus AIP could be classified with an ROC AUC value of 0.99 (Fig. 3B), with a sensitivity and specificity of 97% and 91%, respectively.
Deciphering of Pancreatic Cancer Biomarkers

To better reflect the clinical reality, we then investigated whether differences could be identified between PDAC and the combined, heterogeneous patient group of chronic pancreatitis + AIP + N (n = 103). The results showed that 47 serum proteins were differentially expressed (P < 0.05; Fig. 3A). A majority of these molecules (45 of 47) were found to be upregulated in PDACs. On the basis of unfiltered data, the results showed that PDACs could be distinguished from this heterogeneous patient group with an ROC AUC value of 0.85 (Fig. 3B).

In an attempt to validate the array data, an independent 10-plex MSD was applied (Fig. 3C). However, in a majority of the samples, only IL-8 of the 10 targeted serum molecules was above the limit of detection of the MSD assay. Still, the observed upregulation of IL-8 in PDAC versus N, chronic pancreatitis, and AIP, as well as the combined cohort thereof was statistically confirmed (P < 0.05) by the MSD assay in all cases, except for PDACs versus AIP.

**Refined biomarker signature for PDAC diagnosis**

To test the strength of the classification on all patients, including PDACs, N, chronic pancreatitis, and AIP (n = 103), we first split the cohort into a training set (two thirds of the patients) and test set (one third; Fig. 4A). Next, a condensed serum biomarker signature composed of the 25 nonredundant molecules contributing the most to the classification in the training set was deciphered using a backward elimination strategy. This 25-analyte condensed biomarker signature composed of, for example, cytokines and complement proteins is shown in Fig. 4B. On the basis of the observed SVM decision values for the 25-analyte signature, a power analysis showed that a sample size of 9 patients per group in the test set was required to reach a statistical power of 80% (see Supplementary Material and Methods). Our test set contained 11 patients with PDACs and 24 non-PDAC patients, corresponding to an estimated statistical power of 86%. We then applied this 25-analyte classifier on the independent test set (Fig. 4C). The data showed that PDAC could be diagnosed with an ROC AUC value of 0.88 (Fig. 4C) and with a sensitivity and specificity of 73% and 75%, respectively. Next, we evaluated whether CA 19-9, known to be associated with PDACs, could improve the classification of this 25-analyte panel. To this end, the levels of CA 19-9 were determined with a commercially available ELISA. The results showed that the ROC AUC decreased to 0.81 when including the CA 19-9 data.

To further challenge the classifier in silico, we statistically evaluated its discriminatory power. First, 1,000 different, random signatures of the same length (25 biomarkers) were generated in the training set and applied on the test set. The results showed that the AUC values for the random signatures were lower than that of the identified classifier biomarker signature (AUC = 0.88) in 95% of the cases (Fig. 4D). In addition, the AUC values for a 25-biomarker signature selected on the basis of either lowest P values (AUC = 0.77) or highest fold changes (AUC = 0.78) were lower than that of the identified classifier signature. Hence, the data further indicated the discriminatory power of our classifier, and the strength of the backward elimination strategy for defining a condensed, high-performing signature without overfitting the data. Furthermore, the sample annotation of the test set was...
permutated 1,000 times to compare the specific classification with random classification. The results showed that a higher AUC value (0.88 vs. 0.19–0.86, median value of 0.5) was obtained when the correct sample annotation was used, as compared with the permuted annotation, further showing the strength of the deciphered classification.

Discussion

Pancreatic cancer has a dismal prognosis mostly due to an advanced stage at the time of diagnosis. One way to improve prognosis would be to find PDACs at an earlier stage when the tumor is small and still confined to the pancreas and has not metastasized. However, the current diagnostic tools are unable to perform accordingly and no high-performing biomarker(s) for PDACs are yet available (2, 24). We have recently described an affinity proteomic approach that allowed us to identify several serologic biomarker signatures, distinguishing other cancer indications from healthy controls, showing the strength of the technology platform (17, 18, 22, 25). On the basis of the notion that immunoregulation is a particular phenomenon in PDACs (26, 27), we thus undertook an affinity proteomics approach to harness the diagnostic power of the immune system to diagnose PDACs. To this end, we designed our antibody microarray to target predominantly some of the key regulatory molecules found in serum.
With a limited range of specificities ($n = 57$), PDAC-specific candidate biomarker signatures with high diagnostic power could be identified. Consequently, we showed that serum harbors sufficient information to discriminate between not only well-defined patient cohorts of PDACs versus healthy controls and pancreatitis, respectively, but also between PDACs and the combined cohort of controls and patients with pancreatitis still with high accuracy. This latter finding is particularly critical, as a candidate biomarker signature must do well also in the clinical setting, where heterogeneous patient groups will be tested.

Several risk factors have been reported for PDACs, including smoking and health history (e.g., diabetes and certain hereditary conditions), as well as gender, race, age, obesity, diet, and alcohol. How and if such factors impact the defined biomarker signature(s) is not previously known and would have to be analyzed factor by factor, representing major efforts beyond the scope of this biomarker discovery/prevalidation study.

It has been observed that smoking, a risk factor for many diseases, increases the risk of developing PDACs with a factor of about 2. Although the smoking history was not available for the patients included in this study, we still tried to elucidate whether smoking per se was a confounding parameter. Consequently, we applied both the 18 and 25 signatures on a novel independent cohort of general patients ($n = 64$), for which the smoking history was known (data not shown). Assuming that smoking was a confounding factor, we hypothesized that the signatures would be able to classify these 64 patients based on their smoking history. This was not found to be the case ($AUC \leq 0.55$), which indicated that smoking should not be a confounding factor in the present study. Furthermore, attempts to classify nonsmokers versus smokers in the general patient cohort, based on differentially expressed analytes between these 2 subgroups also failed, resulting in an ROC AUC of 0.50.

In the quest for cancer biomarkers, systemic inflammation is frequently highlighted as a potential confounding factor (28), as cancer development and inflammation have been reported to be associated. In early studies based on affinity proteomics, the results often showed that general disease (inflammatory) signatures, rather than cancer-specific fingerprints, were identified (29–31). Notably, we showed herein that PDAC and pancreatitis could be specifically discriminated with high confidence. Furthermore, the observed signature(s) showed significant differences (only 4–11 analytes overlapped) with those observed for various inflammatory conditions, such as systemic lupus erythematosus, systemic sclerosis, preeclampsia, and Helicobacter pylori infection (25, 32, 33), as well as other types of cancers, such as breast cancer, gastric adenocarcinoma, and glioblastoma multiforme (17, 18, 22, 25). Consequently, this further supported the current observation that
we had defined a PDAC-associated signature rather than a general cancer or inflammatory signature. However, a certain overlap was expected, as the biomarker space searched in these studies (≤65 analytes) was limited. Of note, one should be careful when comparing signatures merely by comparing the overlap, as this does not reflect whether the analytes are up- or downregulated. It is the unique combination of markers combined with the levels of each of them that make up the discriminatory power of a biomarker signature. Furthermore, many analytes display pleiotropic functions, making it difficult to explain their role in a specific disease or whether they, in fact, are part of a systemic response against the disease.

The identified serum immune signatures could be considered snapshots of the immunologic activity in a patient at the time of sampling. Hence, these fingerprints reflect a combination of indirect (systemic) effects in response to the cancer, as well as factors secreted by the tumor. Previous reports have shown that PDAC cell lines expressed a set of cytokines, including, for example, IL-6, IL-8, IL-10, IL-12, IL-13, IL-18, and TGF-β1 (34). Furthermore, several of these and other cytokines, for example, VEGF and IL-7, have also been found to be overexpressed in PDAC tissue, serum, and/or plasma (34–38). This is in keeping with our current observations. Although cytokines play a pivotal role in the immune system, interpreting these intricate expression patterns in a biologic context is demanding, as many of these biomolecules display pleiotropic functions. PDAC, in particular, is characterized by irregular cytokine expression patterns (34). While the expression of, for example, IFN-γ could signal a Tα1 cell-dependent antitumor response (34), the immunologic state in patients with PDACs has often been found to be immunosuppressive, as illustrated by the concomitant expression of anti-inflammatory cytokines, for example, TGF-β and IL-10, and potentially proinflammatory cytokines, such as IL-12 and IL-18 (34). A cellular immunosuppressive state is a distinct biologic feature of PDACs, observed in many patients (39). While Tα2-skewed responses have been reported, the Tα1/Tα2 balance, as indicated in the present study, has also been observed (34, 36, 40). The cytokine expression pattern in PDAC has also been reported to reflect other parameters, such as survival (17, 34). Furthermore, several complement proteins, such as C3, have been suggested to function in immune surveillance against tumors (41, 42), and the carbohydrate antigen Lewis X has also previously been found to be associated with PDACs (43), although its biologic role remains to be fully clarified.

The impact of a high-performing PDAC classifier with high predictive power would be of great clinical significance, as no validated serologic discriminator is yet available (3, 9–11, 44, 45). The present data outlined a potentially added clinical value, as the median sensitivity (88%) and specificity (85%) of our PDAC classifier was significantly higher, compared with what have been consistently showed for CA 19-9 (3, 11, 13, 14). Noteworthy, adding CA 19-9 to our classifier did not improve the prediction. Whether other single biomarkers, suggested to be associated with PDAC, such as CRP, CA 242, GDF-15, haptoglobin, M2-pyruvate kinase, serum amyloid A, platelet factor 4, and IGFBP-1, could improve the discriminatory power of our novel biomarker panels needs to be elucidated. Furthermore, we have recently modeled the impact of new diagnostic possibilities on cost, survival, and quality of life for risk patients and showed that affinity proteomics has a great possibility to become a cost-effective tool in the diagnosis and screening of patients at risk for developing PDAC (Borrebaeck and colleagues, unpublished observations).

From a necessity point of view, only patients with an established diagnosis of PDACs (stage III or IV) were included in our study, as in most PDAC biomarker studies, thus representing patients with clinically manifested cancer. Also, as almost all patients with PDACs sooner or later have their recurrence, we cannot, from current data, tell whether the signature represents a disseminated disease among these patients. This represents a potential limitation, however, future screening studies can only be conducted when a biomarker signature has been developed and validated in patients with PDACs. Hence, the signatures will serve as the starting point for early diagnosis by targeting more suitable clinical samples, such as serum samples collected before current diagnosis, that is, patients with early-stage PDACs (stage I or II). The classifier will perform at its best if early diagnosis, that is, when the tumor is still small and operable, could be achieved (3, 11). Ideally, it would then enhance diagnostic accuracy in, for example, patients with mass-forming lesions in chronic pancreatitis and patients with mucinous cystic lesions of the pancreas. The ultimate goal would be to detect even early premalignant lesions, such as intraductal papillary mucinous neoplasia or ductal pancreatic intraepithelial neoplasia (8). This would then give us the possibility for surveillance of high-risk individuals, such as patients with familial pancreatic cancer or hereditary pancreatitis (46). Even patients with a newly onset diabetes type II lacking risk factors or family history, could be screened.

Taken together, we have addressed a highly unmet medical need and shown that immunosignatures represents a powerful indicator, which allowed identification of the first prevalidated serologic biomarker signature for pancreatic cancer diagnosis. This was achieved with a high-performing microarray platform, well-controlled samples, and stringent bioinformatic and validation approaches. The predictor signature will be further validated in prospective follow-up studies, in which extended independent sample cohorts will be profiled. In the end, these findings will potentially provide improved capabilities for diagnosis and prevention and consequently better prognosis for a devastating disease as pancreatic cancer.

**Disclosure of Potential Conflicts of Interest**

C. Wingren and C.A.K. Borrebaeck have ownership interest in patent application for the biomarker signatures. No potential conflicts of interests were disclosed by other authors.

**Authors' Contributions**

**Conception and design:** R. Andersson, C.A.K. Borrebaeck

**Development of methodology:** C. Wingren, A. Sandström, R. Andersson

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** R. Segersvärd, R. Andersson, M. Lörh

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** C. Wingren, A. Sandström, R. Segersvärd, A. Carlsson, R. Andersson, C.A.K. Borrebaeck

**Writing, review, and/or revision of the manuscript:** C. Wingren, A. Sandström, R. Segersvärd, R. Andersson, M. Lörh, C.A.K. Borrebaeck
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