



Comparing human pancreatic cell secretomes by in vitro aptamer selection identifies cyclophilin B as a candidate pancreatic cancer biomarker

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Most cases of pancreatic cancer are not diagnosed until they are no longer curable with surgery. Therefore, it is critical to develop a sensitive, preferably noninvasive, method for detecting the disease at an earlier stage. In order to identify biomarkers for pancreatic cancer, we devised an in vitro positive/negative selection strategy to identify RNA ligands (aptamers) that could detect structural differences between the secretomes of pancreatic cancer and non-cancerous cells. Using this molecular recognition approach, we identified an aptamer (M9-5) that differentially bound conditioned media from cancerous and non-cancerous human pancreatic cell lines. This aptamer further discriminated between the sera of pancreatic cancer patients and healthy volunteers with high sensitivity and specificity. We utilized biochemical purification methods and mass-spectrometric analysis to identify the M9-5 target as cyclophilin B (CypB). This molecular recognition-based strategy simultaneously identified CypB as a serum biomarker and generated a new reagent to recognize it in body fluids. Moreover, this approach should be generalizable to other diseases and complementary to traditional approaches that focus on differences in expression level between samples. Finally, we suggest that the aptamer we identified has the potential to serve as a tool for the early detection of pancreatic cancer.

Introduction

At the time of diagnosis, the vast majority of patients with pancreatic cancer have locally advanced or distant metastatic disease that is not resectable (1). Earlier detection of the disease could identify more patients at a stage when the tumor is still curable with surgery. A serum-based assay for pancreatic cancer would be an invaluable tool for screening high-risk patients. However, biomarkers such as cancer antigen 19-9 (CA19-9) that are currently being used in clinical practice for monitoring response to therapy lack the sensitivity required to detect the disease at an early stage (2). Additionally, CA19-9 levels cannot be detected in pancreatic cancer patients with Lewis negative (a-, b-) blood type, up to 10% of the population (3). This necessitates the search for better biomarkers that can be employed for the purposes of screening and diagnosis.

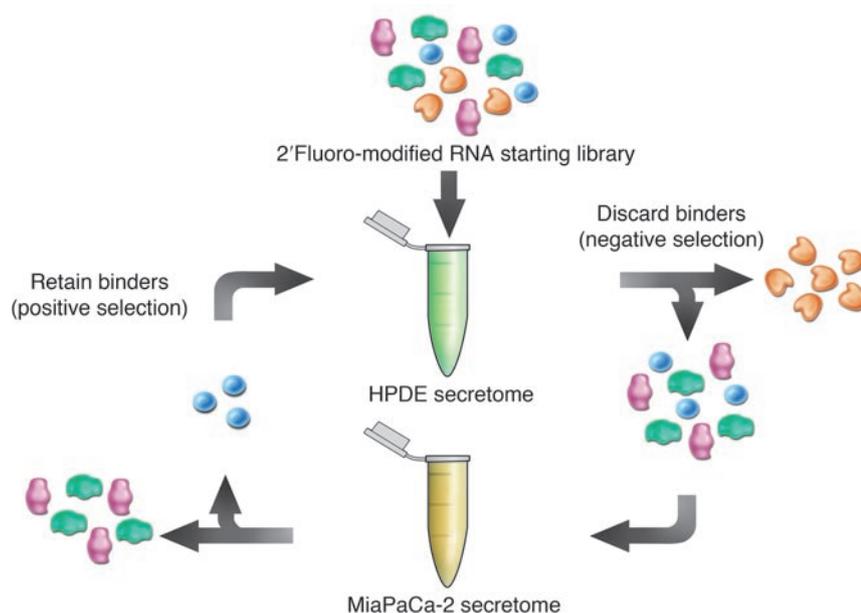
The secretome is the collection of all proteins that are either secreted by a cell or shed from its surface into body fluids and is an obvious source to mine for potential cancer biomarkers (4, 5). The rationale behind using the secretome for biomarker discovery is that cancerous cells secrete proteins that are either qualitatively or quantitatively different from those secreted by normal cells. Proteomic approaches using mass-spectrometric analysis such as stable isotope labeling with amino acids in cell culture (SILAC) (6), multi-dimensional protein identification technology (MudPIT) (7), and surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) (8) have been employed to detect novel biomarkers from conditioned media of pancreatic cancer cell lines. These methods, reliant on peptide

sequence information, have generated lists of proteins that are differentially expressed in the pancreatic cancer secretome. However, in order to validate candidate biomarkers and translate them into clinical detection reagents, time-consuming methods such as antibody development are required. Furthermore, methods reliant on peptide sequence alone will miss aberrant post-translational modifications, conformational changes, or protein complexes that might be present in cancer (9).

Aptamers are oligonucleotide ligands that directly bind their targets with high specificity. By using an in vitro iterative selection process known as SELEX (systematic evolution of ligands by exponential enrichment) (10, 11), RNA and DNA aptamers can be selected that are capable of discriminating between molecular targets with subtle differences, such as proteins with point mutations (12) and small molecules that differ only by a methyl group (13). Several properties of aptamers make them attractive tools for use in a wide array of molecular biology applications. Aptamers bind to their targets with high affinity, demonstrating typical binding dissociation constants (K_d) in the pico- to nanomolar range. They are structurally stable across a wide range of temperature and storage conditions, maintaining the ability to form their unique tertiary structures. Aptamers can be chemically synthesized quickly and relatively cheaply with minimal inter-batch variability, in contrast to the expensive and work-intensive biological systems needed to produce monoclonal antibodies. A variety of nucleotide modifications have been described to increase aptamer stability and affinity (14–16). In particular, SomaLogic Inc. has demonstrated the potential for simultaneous analysis of hundreds of blood proteins using multiplexed modified aptamer assays (15, 17). These aptamers – like most aptamers developed to date – were generated against rationally chosen purified proteins. Therefore, this is

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**Figure 1**

Schema of positive/negative secretome SELEX strategy. An RNA library with a 40-nucleotide random region (greater than 10^{14} potential sequences) and modified with 2'-fluoropyrimidines (2'Fluoro) for nuclease resistance was generated. For negative selection, the RNA library was incubated with the HPDE secretome, and the bound RNAs were removed using nitrocellulose membranes that retained the RNA-protein complexes. The negatively selected unbound RNAs were then incubated with the MiaPaCa-2 secretome. For positive selection, the bound RNAs were similarly partitioned from unbound RNAs and used to generate a pool of RNAs enriched for sequences that bind the MiaPaCa-2 secretome.

a powerful approach for the detection and validation of candidate biomarkers but not for the discovery of new biomarkers.

Instead of applying SELEX to individual molecules, we have applied it to the pancreatic cancer secretome. We devised an *in vitro* “positive/negative” SELEX strategy to identify secreted biomarkers for pancreatic cancer (Figure 1). This method of “blind” selection allows the aptamers to choose their targets. For positive selection, conditioned media representing the secretome of the human pancreatic ductal adenocarcinoma cell line MiaPaCa-2 (ATCC) were used. Conditioned media collected from the immortalized non-cancerous human pancreatic ductal epithelial cell line HPDE-E6E7 (gift of Ming-Sound Tsao, University Health Network, Toronto, Ontario, Canada) (18, 19) were used for negative selection. The positive/negative selection was performed with the objective of selecting aptamers against molecules that are secreted by cancerous cells but not by normal cells. The advantage of this technique lies in its unique potential to simultaneously identify novel biomarkers and develop reagents to detect them in body fluids.

Results

Positive/negative affinity selection to identify biomarkers specific for pancreatic cancer. The MiaPaCa-2 and HPDE6-E6E7 cell lines were initially grown in their respective media to near-confluence, washed, and then incubated for 16 hours in serum-free media (SFM). SFM was chosen because abundant serum proteins could mask the relatively rare proteins secreted by the cell lines. Conditioned media (CM) containing the proteins secreted by the cancerous and non-cancerous cell lines were collected and filtered to remove cellular debris to ensure that the selection was focused against secreted and not cellular molecules. Next, the CM were concentrated using centrifugal spin columns and dialyzed against a salt buffer with pH and electrolyte composition similar to those of serum. The secretomes generated were used for positive/negative *in vitro* SELEX using an RNA library modified with 2'-fluoropyrimidines for nuclease resistance (Figure 1). Standard radioactive filter-based binding assays were used to monitor the progress of selection by measuring the binding of the RNA pools to the secretomes (Fig-

ure 2A). After 9 rounds of positive/negative selection, the RNA pool demonstrated higher binding to both secretomes compared with the starting RNA library but relatively higher binding to the MiaPaCa-2 secretome as compared with the HPDE secretome. The RNA pool from the ninth round was cloned and sequenced. Three dominant sequences were detected upon analysis of the clones and were designated M9-4, M9-5, and M9-6 (Table 1). Individual clones were tested for binding against the MiaPaCa-2 and HPDE secretomes. One of the resulting aptamers (M9-5) demonstrated higher binding to the MiaPaCa-2 secretome compared with the HPDE secretome (Figure 2B). As expected, M9-5 demonstrated higher affinity binding to the MiaPaCa-2 secretome based on the leftward shift of the binding curve relative to the round 9 pool. It is difficult to quantify binding affinity (K_d) in these direct binding assays, because binding does not reach a true asymptotic maximum and because the concentration of the specific protein target within the secretome is unknown. We therefore estimated binding affinity by competition binding assay. M9-5 demonstrated an IC_{50} of 1.5 ± 0.02 nM (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI62385DS1), an affinity suitable for detection of non-abundant serum proteins.

Aptamer M9-5 as pancreatic cancer biomarker. We then evaluated the possibility of using M9-5 as a blood-based biomarker to detect pancreatic cancer. We tested aptamer M9-5 for binding activity in sera collected from human patients with recently diagnosed, radiographically localized pancreatic cancer and from healthy volunteers. Undiluted sera were used without further processing or adjustment for total protein concentration. Examples of binding curves from a cancer patient with high binding activity and a healthy volunteer are shown in Figure 3A. Aggregate binding values for undiluted serum (maximal fraction bound [FB]) from 24 pancreatic cancer patients were significantly higher those than from 24 healthy volunteers (median, 0.25 vs. 0.10, $P < 0.0001$, Figure 3B). No significant differences were seen between subgroups of patients based on age, sex, tumor location, clinical stage, or CA19-9 level, and notably CA19-9 levels were normal (less than 40 U/ml) in 10 (41%) of the cancer patients (Supplemental Table 1). The other

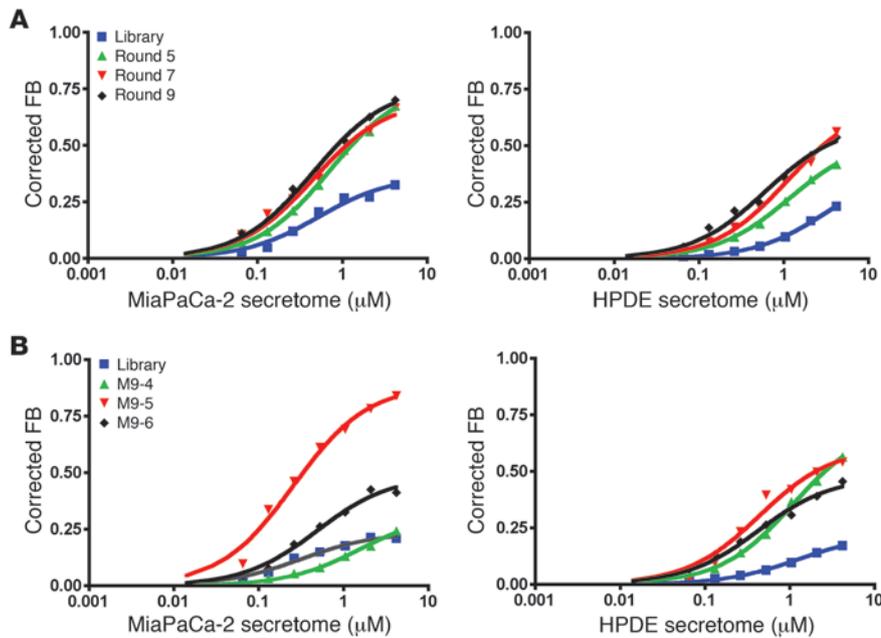


Figure 2

Secretome SELEX generated an aptamer, M9-5, that demonstrated high-affinity binding to MiaPaCa-2 secretome. (A) Binding affinity of the RNA pools for the secretomes, as measured by corrected FB in a radioactive filter-based binding assay, increased over successive rounds of selection. Molecular weight of 50 kDa was arbitrarily chosen to calculate the molar concentration of the secretome. (B) After 9 rounds of selection, the pool was cloned and sequenced. Three dominant sequences were present and designated M9-4, M9-5, and M9-6 (Table 1). Clone M9-5 demonstrated higher binding affinity for the MiaPaCa-2 secretome as compared with the HPDE secretome.

two aptamers, M9-4 and M9-6, demonstrated no significant binding to the pancreatic cancer patient sera (Supplemental Figure 2). Sensitivity and specificity are inversely related and dependent on the threshold for positivity of a test, but — when a maximal FB of 0.14 was selected as a threshold — M9-5 had a false positive rate (1 – specificity) of 4% and a true positive rate (sensitivity) of 92% for discriminating between cancer patients and healthy volunteers. In addition, 3 paired serum samples obtained from cancer patients before and after chemoradiation therapy demonstrated a modest decrease in M9-5 binding, consistent with their response to treatment. All 3 patients demonstrated radiographic stability on restaging but pathologic response to treatment in their subsequent resection specimens, which indicated that M9-5 has the potential to be used as a tool to monitor response to therapy (Figure 3C).

In order to assess whether this marker is species specific, we also tested unprocessed sera from genetically engineered mice with pathologically confirmed pancreatic carcinoma resulting from conditional mutations in *K-ras* and *Trp53* under control of the pancreas-specific promoter *Pdx1* (KPC mice) (20). M9-5 binding in the sera of KPC mice with pancreatic carcinoma was elevated as compared with sera from wild-type mice (median, 0.41 vs. 0.14, $P = 0.002$, Figure 3D). Additionally, the secretome was collected from pancreatic cancer cells isolated and cultured from a KPC mouse tumor (21) and used for the M9-5 aptamer filter-binding assay. M9-5 bound to the KPC secretome with affinity comparable to that of the MiaPaCa-2 secretome (Supplemental Figure 3A).

Identification of the M9-5 target. First, we confirmed that the M9-5 target was protein by demonstrating that binding activity was completely abolished by proteinase K treatment (Supplemental Figure 4). To purify the M9-5 target, we serially fractionated the secretome using the following biochemical purification protocol and retained the fraction(s) with M9-5 binding activity (Figure 4A). M9-5 binding activity was present in the eluate of a cation exchange col-

umn and flow-through of an anion exchange column, indicating that the M9-5 target is a basic protein (Supplemental Figure 5). The eluate from the cation exchange column was next fractionated by molecular weight using gel filtration column chromatography (Supplemental Figure 6A). Several fractions with high binding activity were identified, corresponding to an approximate molecular weight range of 15–30 kDa (Supplemental Figure 6B). Secreted proteins are often glycosylated. Therefore, two fractions with high binding activity were pooled and further fractionated using a wheat-germ-agglutinin (WGA)-lectin column, which binds *N*-acetylglucosamine (GlcNAc) and terminal GlcNAc moieties that are commonly present in many secreted glycoproteins. The flow-through and the eluate were collected and tested for M9-5 binding activity, which was present in the flow-through fraction (Supplemental Figure 7), indicating that the M9-5 target does not possess this specific glycosylation pattern. This purified fraction was separated by gel electrophoresis, revealing an approximately 20-kDa dominant band on Coomassie staining (Figure 4B). The band was excised and subjected to mass-spectrometric analysis. Cyclophilin B (CypB) was identified with 10 unique peptides (46% sequence coverage of secreted CypB) (Supplemental Table 2).

Four other fractions spanning the range of fractions with high binding activity from the gel filtration column were subjected to mass-spectrometric analysis in solution. The rationale for this analysis was that the M9-5 target should be a common protein

Table 1
Sequences of round 9 secretome-SELEX clones

Clone	% of pool	GGGAGGACGAUGCGG-N ₄₀ -CAGACGACUCGCCCGA
M9-4	11	GGUUAGAGGCGACGUCCCGAAUCCGCGCCCAUUGCCCCC
M9-5	28	GGACCUAUGCAGUAGCCAGUGUGGACUGGGCUGCCCCC
M9-6	39	CGAGCGUAGAAUGUCUUFCCACUAGACGUCGUUCGGCCCC

Sequence for the random portion of the aptamers is shown.

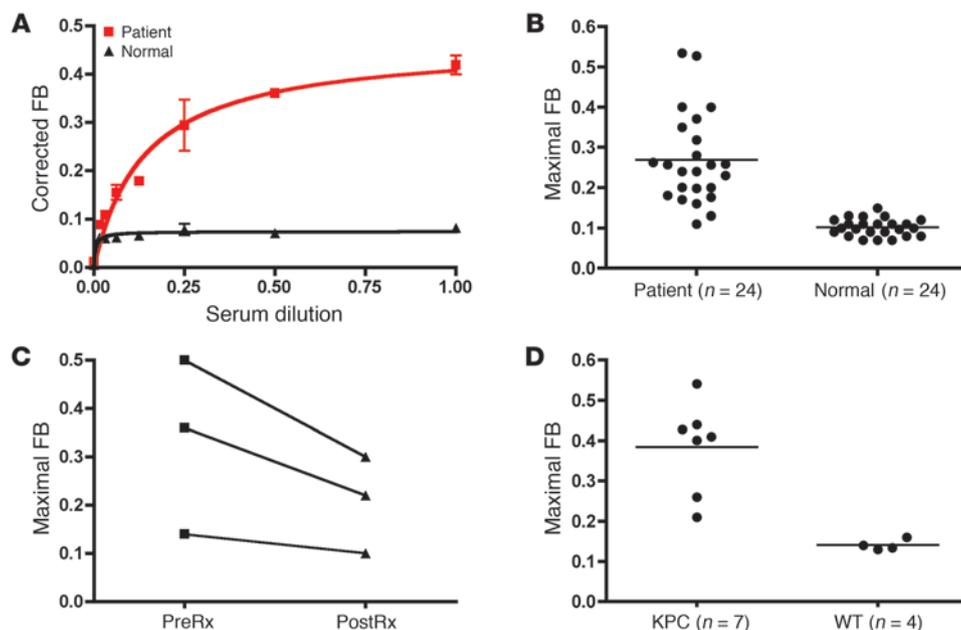


Figure 3

Characterization of M9-5 as a pancreatic cancer biomarker. (A) Examples of full binding curves from a pancreatic cancer patient with high binding activity and a healthy volunteer. Binding assays were performed in duplicate, and the error bars represent SD. (B) The fraction of aptamer bound to undiluted serum was defined as maximal FB and found to be significantly higher in the 24 pancreatic cancer patients than in the 24 healthy volunteers ($P < 0.0001$). Each dot represents the mean of 3 independent binding experiments. (C) Maximal FB decreased in 3 pancreatic cancer patients with blood samples collected before and after neoadjuvant chemoradiation (PreRx and PostRx). (D) Maximal FB was also higher in 7 KPC mice with localized pancreatic cancer than in 4 wild-type mice.

detected in all fractions with M9-5 binding activity. CypB was detected in all fractions with high peptide coverage (Supplemental Table 2). To verify the mass-spectrometric analyses, we subjected the MiaPaCa-2 secretome and WGA column flow-through to Western blot analysis using a rabbit polyclonal anti-CypB antibody, and an approximately 20-kDa band was detected (Figure 4C) that was slightly smaller than recombinant CypB, which possesses a hydrophobic leader sequence not present in the secreted form (22). CypB was similarly detected in the KPC secretome (Supplemental Figure 3B). As further confirmation, we also demonstrated that biotinylated M9-5 aptamer could be used to affinity purify CypB from the MiaPaCa-2 secretome (Figure 4D).

Finally, we used a commercially available ELISA kit to measure CypB levels in the same set of cancer patient and healthy volunteer sera that were analyzed using the radioactive M9-5 binding assay. CypB levels were indeed greatly elevated in the pancreatic cancer patient sera as compared with the healthy volunteer sera (median, 295 ng/ml versus 60 ng/ml, $P < 0.0001$, Figure 5A) similar to the results of the M9-5 binding experiment (Figure 3B). CypB levels by ELISA were significantly correlated with M9-5 binding ($r = 0.65$, $P < 0.0001$, Figure 5B). These data, together with the mass spectrometry and affinity purification data, strongly suggest that CypB is the protein target of M9-5.

Discussion

A noninvasive test for early detection is a “holy grail” of pancreatic cancer research. Proteins that are differentially secreted by cancerous cells may serve as biomarkers for cancer in body fluids

such as serum. We devised a selection strategy that has the potential to simultaneously discover biomarkers and the reagents to detect them in body fluids. We applied a positive/negative in vitro SELEX approach to the secretomes of a pancreatic cancer cell line (Mia-PaCa-2) and a normal pancreatic cell line (HPDE) with the goal of identifying protein molecules that are preferentially secreted by pancreatic cancer cells. Selections against a variety of complex targets have been performed in studies using whole cancer cells in culture (23–27) and whole tumors in vivo (28) to generate aptamers against proteins that are expressed on the cancer cell surface and which may represent novel targets for imaging or therapy. Our selection against secreted proteins resulted in the identification of three dominant RNA sequences with distinct binding targets, including one, M9-5, that preferentially bound the Mia-PaCa-2 secretome over the HPDE secretome. Analysis of sequences from earlier rounds will likely yield a greater number of aptamers and possibly other candidate biomarkers.

When tested against human sera, the M9-5 binding assay demonstrated excellent potential to discriminate between patients with known pancreatic cancer and healthy volunteers. However, the most important measures of a screening test are its positive and negative predictive values, which are dependent on the proportion of positives in the population tested. Therefore, a much larger study — including patients with benign diseases of the pancreas — is necessary to appropriately compare the performance of this assay to use of established tumor markers such as CA19-9.

In order to identify the M9-5 target, we serially purified the Mia-PaCa-2 secretome using a variety of biochemical methods and followed the binding activity. At the end of the final purification step, the fraction was resolved by gel electrophoresis, and an approximately 20-kDa major polypeptide band was identified as CypB by mass-spectrometric analysis. To verify the M9-5 target identification, we used biotinylated M9-5 aptamer for affinity purification, and CypB was detected upon Western blot analysis of the purified proteins. As further confirmation, we measured CypB levels using ELISA in the same human serum samples tested for M9-5 binding and found a statistically significant correlation between M9-5 binding and CypB levels by ELISA.

CypB is an ER-resident protein that possesses peptidyl-prolyl *cis/trans*-isomerase activity (29, 30) and is known to be secreted into body fluids (31). It has been implicated as a tissue biomarker in other cancers (32–35) and was also previously detected using a more traditional proteomics approach as one of several proteins secreted from the pancreatic cancer cell line Suit-2 (7), but CypB has not previously been evaluated as a serum cancer biomarker.

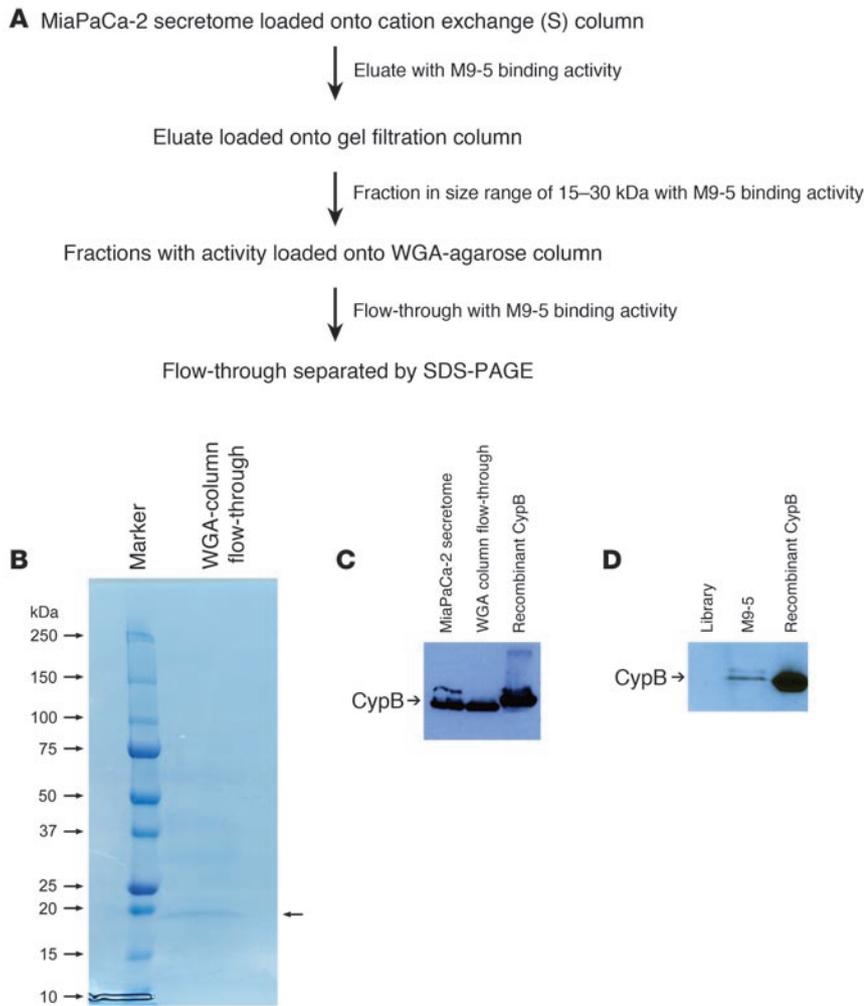


Figure 4

Purification and identification of CypB as the M9-5 target protein. **(A)** Schema for the biochemical purification of the M9-5 binding target from the MiaPaCa-2 secretome. **(B)** Resolution of the WGA-agarose column flow-through, the final purified fraction, by SDS-PAGE revealed a single dominant band of approximately 20 kDa on Coomassie staining (indicated by the arrow). Mass-spectrometric analysis identified this band as CypB with high peptide coverage (Supplemental Table 1). Raw data are available for download at <https://proteomecommons.org/>. **(C)** CypB was confirmed to be present by Western blot analysis of WGA-agarose column flow-through (0.2 μg total protein loaded) and MiaPaCa-2 secretome (4 μg total protein loaded). Recombinant CypB (0.01 μg loaded) was used as positive control. **(D)** Western blot analysis using CypB antibody of the proteins pulled down by a biotinylated version of the M9-5 aptamer revealed a 20-kDa band. No bands were detected when the starting RNA library was used for affinity purification.

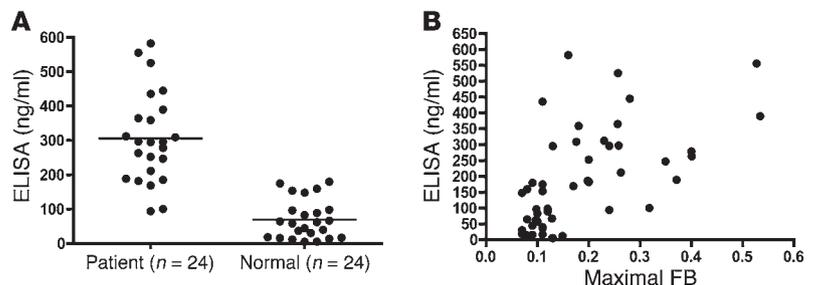
Since the vast majority of pancreatic cancers arise from exocrine cells of the pancreas, it is interesting that CypB has been detected in rat zymogen granules (ZGs) (36). ZGs are specialized storage organelles of pancreatic acinar cells and store inactive pancreatic digestive enzymes that are secreted in response to neuronal or hormonal stimuli. Uncontrolled proliferation of ductal epithelial cells and/or their aberrant architecture in pancreatic cancer might result in the secretion of CypB into the blood. It has also been shown that oxidative stress causes secretion of CypB by vascular smooth muscle cells (37). Since we grew the cells in SFM for collecting the secretome, which is known to lead to oxidative stress (38), it is tempting to speculate that serum starvation of the cells

led to release of CypB into our secretome and that the hypoxic tumor environment *in vivo* (39) may similarly lead to release of CypB into the bloodstream.

CypB may therefore be a sensitive marker of pancreatic pathology, arguably the most important characteristic of a screening test for a deadly disease. Our current efforts are focused on the validation of CypB as a biomarker, using serum samples from human patients with early-stage pancreatic cancer and benign pancreatic diseases. We also found that M9-5 cross-reacts with mouse CypB protein when tested in the KPC mouse model. KPC mice develop *in situ* tumors that recapitulate the different stages of pancreatic ductal adenocarcinoma in humans (20). M9-5 demonstrated

Figure 5

Detection of CypB in serum samples by ELISA. **(A)** CypB levels were measured by ELISA in the same human serum samples analyzed for M9-5 binding activity and found to be significantly higher in the 24 pancreatic cancer patients than in the 24 healthy volunteers. **(B)** There was a significant correlation between M9-5 maximal FB and CypB levels by ELISA.





greater binding to sera collected from KPC mice with pathologically confirmed pancreatic adenocarcinoma than to sera from normal mice. Species cross-reactivity will facilitate the evaluation of CypB as a biomarker in animal models, where tumor burden can be more easily measured and manipulated than in humans.

In parallel, we are working on the comparison and optimization of aptamer- and antibody-based assays for CypB. The imperfect correlation between M9-5 binding and CypB levels by ELISA suggests that the aptamer and antibody may not be binding exactly the same epitopes, and the assays may differ in their ability to discriminate between cancer and benign pancreatic diseases. The aptamer may be a valuable tool for purification of CypB and associated proteins in order to investigate whether there are differences between CypB secreted by cancerous and non-cancerous cells. Meanwhile, the radioactive aptamer filter-binding assay used in this proof-of-concept study is quite simple, but it is not practical for large-scale clinical applications. Furthermore, maximal FB as measured with this assay does not have a linear relationship with protein concentration and may therefore not be the ideal way to quantify CypB levels in body fluids. Fortunately, the relative ease with which aptamers can be chemically modified has led to the development of a variety of aptamer-based detection assays, including surface plasmon resonance (SPR) biosensor assays, which have been used for the detection of picomolar concentrations of protein biomarkers (40). As just one possible alternative, biotinylated M9-5 aptamer can be immobilized on SPR chips coated with streptavidin, an approach that is conducive to the high-throughput analysis of clinical samples and also potentially to multiplex analysis with several aptamer biomarkers.

Traditional proteomic approaches to biomarker identification utilize quantitative differences in expression levels between cancerous and non-cancerous proteomes (6–8). Such approaches generate large sets of data that require subsequent bioinformatics-based approaches to identify and prioritize candidate biomarkers. SELEX basically probes a complex mixture of proteins with a complex library of oligonucleotide shapes and has the potential to detect both quantitative and qualitative differences between cancerous and non-cancerous proteomes. Unlike most mass-spectrometric techniques, our approach – which is based upon molecular recognition – can in principle identify biomarkers that may be missed by standard expression profiling methods and yield aptamers against cancer-specific protein complexes, alternately folded proteins, or post-translational modifications. Critical to this claim is that our approach is not dependent on precise target identification, since the aptamers generated can be used themselves as detection reagents, with chemical properties that may offer technical advantages over antibodies. Although we have utilized this technique to identify secreted biomarkers for pancreatic cancer, it can conceivably be applied to any other cancer or disease for which appropriate proteomes exist for positive/negative selection. Therefore, secretome SELEX is a practical and generalizable approach that can complement bioinformatics-based proteomic approaches to biomarker discovery.

Methods

Preparation of secretome. MiaPaCa-2 cells (ATCC) and HPDE-E6E7 cells (gift of Ming-Sound Tsao, University Health Network, Toronto, Ontario, Canada) (18, 19) were grown in their respective media to near confluence, then washed in their respective SFM and incubated in the SFM for 16 hours. CM were collected and centrifuged at 1,000 g, 4°C, followed by filtration using

a 0.2- μ m sterile filter system (Nalgene) to remove floating cells and cellular debris. Next, the CM were concentrated at 4°C using centrifugal spin columns (VIVA SPIN 20, 3,000 molecular weight cutoff [MWCO], Sartorius Stedium). The concentrated CM were dialyzed against buffer F (20 mM HEPES pH 7.5; 150 mM NaCl; 2 mM CaCl₂ and 3 mM MgCl₂) overnight at 4°C using dialysis cassettes (3,500 MWCO, Thermo Scientific). Total protein concentration of the secretome was determined by using protein assay reagent (Bio-Rad). Aliquots of concentrated CM (secretome) were stored at –80°C for subsequent assays.

Secretome SELEX. Positive/negative selection against the secretome was performed using SELEX protocols as previously described (41) with modifications. The specific DNA oligonucleotide sequences used are described in Supplemental Methods. Briefly, for each round of negative selection, 2 nM RNA was incubated with 50 μ l (1.7 μ g/ μ l) HPDE secretome in buffer F. The final reaction mixture volume was 200 μ l and was incubated at 37°C for 15 minutes. Protein-RNA complexes were partitioned from the unbound RNA pool reaction mixtures using 25-mm, 0.45- μ m nitrocellulose membranes (Whatman). The negatively selected, unbound RNA pool was then incubated with 100 μ l (0.2 μ g/ μ l) MiaPaCa-2 secretome in buffer F at 37°C for 15 minutes. The bound protein-RNA complexes were again partitioned from the unbound RNA pool as described above, except that the protein-RNA complexes on the nitrocellulose membrane were retained. The bound RNA was then extracted from the membrane, reverse transcribed, and PCR amplified to generate DNA templates for the next round of positive/negative selection. After 6 rounds of selection, we decreased the amount of MiaPaCa-2 secretome (to 30 μ l at the same concentration) to increase the stringency of selection. The PCR products of the ninth round of selection were cloned, sequenced, and used as templates for generation of clonal RNA transcripts.

Radioactive filter binding assay. The pooled-round or clonal RNA was dephosphorylated and labeled with 5'-[γ -³²P]-triphosphate as described previously (41). The end-labeled RNA was then used in the double-filter nitrocellulose-binding assay (42). Briefly, the secretome or unprocessed serum was serially diluted in buffer F, and equal amounts of labeled RNA (5,000 cpm, corresponding to a final RNA concentration less than 0.3 pM) were added to each protein dilution at 37°C. The reactions were loaded onto a vacuum manifold containing a nitrocellulose membrane (Whatman) placed over a nylon membrane (PerkinElmer). The membranes were exposed to a phosphorimager screen, scanned, and quantitated with a Storm 825 Phosphorimager (GE Healthcare). The corrected FB was calculated by dividing counts on the nitrocellulose by the total counts and adjusting for background.

Collection of sera. Blood samples from pancreatic cancer patients and healthy volunteers were collected with approval from the Duke University Institutional Review Board. Blood samples were collected in red top Vacutainer tubes (BD Vacutainer), incubated for 45 minutes at room temperature for clotting, then centrifuged at 1450 g, 4°C, for 15 minutes. The supernatant serum was collected and either used immediately or stored at –80°C in aliquots for subsequent assays.

KPC mouse model. *LSL-Kras^{G12D/+}*, *LSL-Trp53^{R172H/+}*, and *Pdx-1-Cre* mice were obtained through the National Cancer Institute's (NCI) Mouse Models of Human Cancer Consortium. KPC mice (20) were bred with the assistance of the Duke Division of Laboratory Animal Resources Breeding Core. The project was approved by the IACUC of Duke University. Mice were evaluated by serial abdominal examination for the development of a palpable abdominal mass, at which point mice were euthanized, and blood was immediately collected in 1.5-ml Eppendorf tubes from the inferior vena cava. The collected blood was incubated at room temperature for 30 minutes for clotting, then centrifuged at 2,000 g, 4°C, for 10 minutes. The supernatant serum was collected and either used immediately or stored at –80°C in aliquots for subsequent assays.



Purification of the M9-5 target. The MiaPaCa-2 secretome was loaded onto cation (S) and anion (Q) exchange spin columns (Thermo Scientific) pre-equilibrated with buffer F. The columns were washed twice with buffer F, and the bound proteins were eluted with high-salt (1,000 mM NaCl) buffer G. Buffer G of the eluate was exchanged with buffer F using a spin column (Amicon Ultra, 3000 MWCO, Sigma-Aldrich). The column flow-through and eluate were analyzed for M9-5 binding activity using the radioactive filter-binding assay described above. The fraction with binding activity was next loaded onto a gel filtration Superose 6 10/300 GL column (GE Healthcare), and fractions were collected using ÄKTApurifier (GE Healthcare) fast protein liquid chromatography (FPLC) following the manufacturer's instructions. Fractions with binding activity were pooled together and loaded onto a WGA-agarose column (Thermo Scientific). The flow-through and eluate were collected, and the buffer was exchanged with buffer F. The purified fraction with binding activity was resolved using 4%–20% SDS-PAGE (Bio-Rad) and stained with Coomassie Blue G-250. Mass-spectrometric analysis was performed as described in Supplemental Methods.

CypB Western blot analysis. Proteins were resolved using 4%–20% SDS-PAGE, then transferred electrophoretically to polyvinylidene fluoride membranes. The washed membranes were then analyzed by Western blotting by using a rabbit polyclonal anti-CypB antibody (1:5,000 dilution; Abcam) with a goat anti rabbit-HRP secondary antibody (1:1,000 dilution; Invitrogen). The signal was detected by the Pierce ECL Western Blotting Substrate (Thermo Scientific). Recombinant CypB (ProSpec) was used as a positive control.

Affinity purification of CypB. M9-5 and starting library RNA (50 pmol) were biotinylated using the RNA 3' End Biotinylation Kit (Pierce). The biotinylated RNAs were incubated with 50 µl (0.2 µg/µl) MiaPaCa-2 secretome at 37°C for 15 minutes. Dynabeads M-280 Streptavidin beads (Invitrogen) prewashed in buffer F were added to the reaction mixture and incubated at 37°C for 10 minutes to capture the biotinylated RNA-protein complexes. The bead-RNA-protein complexes were separated using a Dynal magnet and washed 3 times in buffer F containing 0.01% Tween-20. The proteins bound to the aptamer were eluted by incubating the complex with high-salt buffer G at 37°C for 10 minutes, then resolved by 4%–20% SDS-PAGE and subjected to CypB Western blot analysis.

CypB ELISA. CypB levels in the sera were measured using an ELISA Kit (USCN Life Science Inc.). Serum samples were diluted (1:50) in phosphate-buffered saline, and 100 µl of the diluted samples were used for assay following the manufacturer's protocol.

Statistics. The Mann-Whitney *U* test with a two-sided α of 0.05 was used to compare both M9-5 binding and CypB levels by ELISA between pancreatic cancer patients and healthy volunteers. A Spearman's rank correlation coefficient was calculated to evaluate the relationship between the two values. A receiver operator characteristic (ROC) curve was generated for M9-5 binding by plotting the fractions of true positives (pancreatic cancer patients) and false positives (healthy volunteers) for each threshold value. All statistical analyses were performed in Prism 4 (version 4.0b for Macintosh).

Study approval. Blood samples from pancreatic cancer patients and healthy volunteers were collected with prior written informed consent and approval from the Duke University Institutional Review Board. The KPC mouse protocol was approved by the IAUCUC of Duke University.

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