Published in final edited form as:

J Proteome Res. 2013 June 7; 12(6): 2791–2804. doi:10.1021/pr400139c.

Target Proteomic Profiling of Frozen Pancreatic CD24+ Adenocarcinoma Tissues by Immuno-Laser Capture Microdissection and Nano-LC-MS/MS

Jianhui Zhu, Song Nie, Jing Wu, and David M. Lubman*

Department of Surgery, University of Michigan Medical Center, Ann Arbor, MI 48109

Jianhui Zhu: jianhuiz@umich.edu; Song Nie: songn@umich.edu; Jing Wu: jingwu@umich.edu; David M. Lubman: dmlubman@umich.edu

Abstract

Cellular heterogeneity of solid tumors represents a common problem in mass spectrometry (MS)-based analysis of tissue specimens. Combining immuno-laser capture microdissection (iLCM) and mass spectrometry (MS) provides a means to study proteins that are specific for pure cell subpopulations in complex tissues. CD24, as a cell surface marker for detecting pancreatic cancer stem cells (CSCs), is directly correlated with the development and metastasis of pancreatic cancer. Herein, we describe an in-depth proteomic profiling of frozen pancreatic CD24⁺ adenocarcinoma cells from early stage tumors using iLCM and LC-MS/MS, and a comparison with CD24⁻ cells dissected from patient-matched adjacent normal tissues. Approximately 40 nL tissue was procured from each specimen and subjected to tandem MS analysis in triplicate. A total of 2665 proteins were identified, with 375 proteins in common that were significantly differentially expressed in CD24⁺ versus CD24⁻ cells by at least a 2-fold change. The major groups of the differentially overexpressed proteins are involved in promoting tumor cell migration and invasion, immune escape and tumor progression. Three selected candidates relevant to mediating immune escape, CD59, CD70 and CD74, and a tumor promoter, TGFBI, were further validated by immunohistochemistry analysis on tissue microarrays. These proteins showed significantly increased expression in a large group of clinical pancreatic adenocarcinomas but were negative in all normal pancreas samples. The significant coexpression of these proteins with CD24 suggests that they may play important roles in the progression of pancreatic cancer and could serve as promising prognosis markers and novel therapeutic targets for this deadly disease.

*To whom correspondence should be addressed. David M. Lubman, Department of Surgery, University of Michigan Medical Center, 1150 West Medical Center Drive, Building MSRB1, Rm A510B, Ann Arbor, MI 48109-0656. dmlubman@umich.edu.

Supporting Information Available: Characteristics of frozen pancreatic adenocarcinoma and patient-matched adjacent normal tissue specimens used in this study (Table S1). Proteins identified from CD24⁺ PDAC and CD24⁻ ANT cells across the 6 specimens with FDR < 0.01 (Table S2). Clinical pathologic characteristics of pancreatic adenocarcinoma samples (n = 40) in the tissue microarray (Table S3). Summary of the number of proteins, unique peptides, and total spectral counts identified in single analyses of CD24⁺ PDAC (n = 3) and CD24⁻ ANT (n = 3) cells, respectively (Table S4). Significantly differentially expressed proteins identified in common between CD24⁺ PDAC and CD24⁻ ANT cells (Table S5). List of significantly up-regulated proteins identified in CD24⁺ PDAC cells which were reported previously as overexpressed in the pancreatic cancer proteome with validation evidence (Table S6). Summary of IHC analysis of CD59, CD70, CD74, TGFBI, and CD24 expressions in 8 normal pancreas tissues and 40 pancreatic adenocarcinomas (Table S7). Representative images of IHC double staining with CD44 (green) and CD24 (red), showing less than 1% overlap between the two markers in PDACs (Figure S1). A representative base peak chromatogram of CD24⁺ PDAC samples and MS/MS spectrum of a peptide from CD74 (Figure S2). The network of Cellular Growth and Proliferation constructed by IPA (Figure S3). Representative images of IHC double staining of CD74 (green) with NFkB (red) and CD3 (red), respectively (Figure S4).

Keywords

pancreatic cancer; CD24; frozen tissue; laser capture microdissection; LC-MS/MS; proteome profiling; spectral count

Introduction

Pancreatic ductal adenocarcinoma (PDAC), which accounts for 90% of pancreatic cancers, is the fourth most lethal cancer in the United States.¹ Extensive efforts have been devoted to improve early detection and effective treatment of this highly aggressive cancer. In-depth molecular analyses, which can reveal the alterations that are crucial for the development and maintenance of pancreatic carcinogenesis, provide a means for discovery of novel diagnostic markers and therapeutic targets.² Tissue-based proteome analysis is inherently attractive since the tissues are obtained from the primary site of pathology. This could result in an understanding of the cellular and molecular interactions that drive disease within the tissue and deliver biomarker information with clinical relevance.

Fresh frozen patient-derived tissues have been widely used for proteomic studies of pancreatic cancer and can provide more relevant data than the formalin-fixation and paraffin-embedding (FFPE) tissues. Recently, Turtoi *et al.* investigated the proteomic profiles of three frozen normal pancreas tissues and three tumor lesions using 2D-LC MS/MS, revealing 422 upregulated proteins in the tumor, of which 3 novel overexpressed proteins were confirmed in human PDAC.³ However, a common problem that arises with tissue-based proteomics is the heterogeneous nature of tissues. PDAC typically presents as a solid mass characterized by a low percentage of tumor cells (< 40%) embedded in a densely desmoplastic stroma.⁴ The heterogeneity of the tissue can have a confounding effect on proteomics studies and limit discovery of tumor specific biomarkers. Therefore, molecular profiling of a pure and homogeneous cell population is essential for correlating molecular signatures in cancer and cancer-free cells.

Laser-capture microdissection (LCM) is a popular approach for directly procuring pure cell subpopulations under microscopic visualization, which can overcome the problem of tissue heterogeneity⁵ and permit the isolation of malignant, premalignant and normal cells from a complex tissue.⁶ A study demonstrated that enrichment of normal and malignant pancreatic ductal epithelial cells from clinical tissue specimens by LCM facilitated the direct comparison of protein profiles between the normal and tumor cell types.⁷ Another study showed that combining LCM of malignant epithelia from primary PDAC and matched lymph node metastatic tissues with downstream proteomic analysis provided a valid approach for better understanding of PDAC metastatic spread.⁸ Furthermore, together with immunohistochemical antibody-staining, the immuno-LCM (iLCM) can detect target cell populations with a specific immuno-phenotype from complex tissues and therefore enables a more accurate molecular profile.⁹ This is especially useful where one might be interested in various sub-populations of cells that might exist, such as in the cancer stem cell population.

CD24 plays an important role in the tumorigenesis and progression of pancreatic cancer. It is a candidate protein for detecting cancer stem cells (CSCs) in PDAC, which possess the ability to both self-renew and produce differentiated progeny.¹⁰ CD24, as a single marker, exhibits highly tumorigenic potential, and as few as 500 primary CD24⁺ PDAC cells were capable to induce tumor formation in xenograft models.¹⁰ Furthermore, CD24 has been reported to stimulate tumor cell proliferation, promote tumor cell binding to P-selectin, and strongly induce cell motility and invasion,¹¹ implicating CD24 in the regulation of tumor growth and metastasis.¹² Patients with PDAC have a doom prognosis when these tumors

were previously shown to have a high level of CD24.¹³ Taken together, these observations suggest that studies of CD24⁺ cells in primary PDAC tissues could provide a means to identify markers involved in pancreatic tumorigenesis and progression.

Herein, we performed an in-depth proteomic analysis of CD24⁺ and CD24⁻ cells procured from frozen PDAC at early stages and patient-matched adjacent normal tissues (ANTs), respectively, by combining iLCM with LC-MS/MS. Considering that PDAC contains a highly desmoplastic stroma and the microenvironment of the local tumor can be an active participant, where stroma and tumor cells exchange enzymes and other molecules to promote proliferation and survival,⁴ we did not collect CD24⁻ cells within PDAC tissues. Instead, CD24⁻ cells were enriched from patient-matched adjacent normal tissues. After isolation of the pure and homogenous cell populations, the protein content was extracted using the filter-aided sample preparation method (FASP), a streamlined approach for rapid and sensitive proteome mapping and highly efficient analysis of lysates from low numbers of cells.¹⁴ The digested peptides were further separated by reverse-phase chromatography and analyzed by tandem mass spectrometry using an Orbitrap Elite. To our knowledge the current work presents the first direct proteome comparison of CD24⁺ PDAC cells and CD24⁻ ANT cells derived from fresh human patient specimens after surgery. The results presented here not only demonstrate the potential of this approach, but could contribute to revealing molecules associated with tumor initiation and progression and an improved understanding of pancreatic tumorigenesis.

Experimental Methods

Immunohistochemistry of Frozen Tissues with CD24

Three patient-matched pairs of frozen pancreatic adenocarcinoma (PDAC) in early stages and adjacent normal tissue (ANT) sections (5 μ m thickness) were obtained from BioChain Institute, Inc. (Newark, CA). Patient and tumor characteristics are listed in Supplementary Table S1. Tissue sections were stored at -80°C and, when used, were immediately hydrated in 70% ethanol for 30 s and rinsed in deionized water for 30 s. They were then incubated with 1% BSA in PBS for 1 hr at room temperature to block non-specific binding, and immunostained with mouse anti-CD24 (Abcam, Cambridge, MA) at room temperature for 1 hr at a dilution of 1:200. The sections were subsequently stained with HRP conjugated anti-IgG (Abcam, Cambridge, MA) for 30 min at room temperature, and then developed in DAB solution (Vector Labs, Burlingame, CA). Tissue sections were washed with PBS for 5 min three times between each step, and all aqueous solutions contained 1 $\times$ protease inhibitor cocktail (Roche). Sections were counterstained with hematoxylin for 15 s to show nuclei, subsequently dehydrated in alcohol (70% ethanol, 95% ethanol, 100% ethanol twice, 30 s each), and then in xylene for 1 min. Tissues were completely dried prior to LCM.

Immuno-Laser Capture Microdissection

LCM was performed on a VeritasTM Arcturus LCM (Arcturus, CA), which has a combined IR/UV system. The CapSure[®] Macro Cap (Applied Biosystems[®]) was placed over the tissue prior to laser microdissection. Once the UV laser cuts the desired region, the IR laser tacks the polymer onto the cap to capture the chosen cells. LCM parameters were: power = 50 mW, pulse duration = 2 ms, and spot size = 20 μ m. Approximately 8.0 mm² surface area, which is equal to 40 nL tissue, was procured per clinical specimen and subjected to subsequent shotgun proteomic analysis. The LCM cell procurement time for each specimen was approximately 2 hr. CD24⁺ and CD24⁻ cells were microdissected from patient-matched PDAC (n = 3) and ANT (n = 3) samples, respectively.

Protein Extraction and Digestion by FASP

Protein extraction and digestion was performed using the filter-aided sample preparation method (FASP). The FASP™ Protein Digestion kit was purchased from Protein Discovery, Inc. (Knoxville, TN). The procedures were performed according to manufacturer's instructions. Microdissected cells were lysed in 25 µL of lysis buffer, which contains 4% SDS and 0.1 M DTT, at 95 °C for 1 h with periodic agitation. The extract was mixed with 200 µL of 8 M urea in 0.1 M Tris-HCl and centrifuged at 14 000 g for 30 min on a spin filter with a molecular weight cut-off (MWCO) of 30 kDa. Then 100 µL of 50 mM iodoacetamide in 8 M urea was added to the filters and incubated with the sample in the dark for 20 min. Filters were subsequently washed twice with 8 M urea, followed by two washes with 50 mM NH₄HCO₃. Enzymatic digestion was performed by adding trypsin (Promega, Madison, MI) in 75 µL of 50 mM NH₄HCO₃ to the filter and incubated overnight at 37 °C. Released peptides were collected by centrifugation and desalted with ZipTip C18™ tips (Millipore, Billerica, MA).

LC-MS/MS

The samples were analyzed in triplicate by LC-MS/MS. LC was performed on a Proxeon Easy-nLC II system (Thermo) with a flow rate of 400 nL/min. Peptides were analyzed by an Orbitrap Elite mass spectrometer (Thermo), separated on a 25 cm column with 75 µm inner diameter, packed in-house with the reverse phase material Magic C18 AQ 100, 5 µm particle size. A 130 min linear gradient was applied from 2 to 35% acetonitrile in 0.1% formic acid. The MS instrument was operated in positive ion mode. Survey MS scans (from m/z 400–2000) were acquired in the orbitrap analyzer with resolution $R = 120\,000$ at m/z 400, and the top 20 most intense ions were selected for tandem MS analysis by CID in the linear ion trap. The high sequencing speed of the Elite instrument allowed employing a “top 20” method for CID, combining survey scans at a high resolving power with collision-induced dissociation of the 20 most abundant precursors, which has been demonstrated to increase protein identifications in complex mixtures by about 30%.¹⁵ The normalized collision energy was set at 35% for MS/MS.

Data Analysis

All MS/MS spectra were searched using SEQUEST algorithm 29 incorporated in Proteome Discoverer software version 1.1.0.263 (Thermo) against the UniProt human protein database Release 2010_11. The initial maximal mass tolerance in MS mode was set to 10 ppm, whereas fragment mass tolerance was set to 0.8 Da. The maximum false peptide discovery rate was specified as 0.01. Carbamidomethyl (C) was set as a fixed modification and oxidation (M) as a variable modification. Label free quantification was carried out using the spectral counting method, which is based on counting the total number of fragmentation spectra assigned to peptides for a given protein^{16–17} and has been shown to be a popular and practical approach for quantitative analysis of proteomic data. All proteins identified in this study are listed in Supplementary Table S2.

Ingenuity Pathway Analysis (IPA)

In order to obtain detailed molecular information and infer significant signaling pathways from the proteome profiling results, differentially expressed proteins identified between CD24⁺ PDAC and CD24[−] ANT cells in three patient-matched sample pairs were uploaded into the pathway analysis tool IPA (Ingenuity Systems®). The uploaded Excel file contains the relevant proteins with their fold change, p -value and corresponding primary accession number. The significance values for canonical pathways were calculated using the right-tailed Fisher's Exact Test by comparing the number of proteins that were involved in a given

function or pathway relative to the total number of occurrences of these proteins in all functional/pathway annotations stored in the Ingenuity Pathway Knowledge Base (IPKB).

Tissue Microarray and Double Immunohistochemical Analysis

The tissue microarrays (TMAs) of formalin-fixed paraffin-embedded pancreatic tumors and normal tissues were purchased from US Biomax Inc. (Rockville, MD). Tissue specimens included 8 normal human pancreas tissues (age: from 21 to 50 years, median: 35 years) and 40 cases of pancreatic adenocarcinomas (age: from 31 to 78 years, median: 56 years) with clinical stages and pathology grades. The clinical characteristics are listed in Supplementary Table S3.

The TMAs were dewaxed in xylene for 10 min twice and rehydrated through a series of alcohol solutions to water. Antigen retrieval was achieved by boiling the arrays in citrate buffer at pH 6.0 (Invitrogen) for 15 min. The TMAs were incubated with 1% BSA in PBS for 1 hr at room temperature to block non-specific binding. To achieve double immunofluorescence staining of CD24 and other protein candidates, mouse anti-CD24 (Abcam, Cambridge, MA) was mixed with rabbit anti-CD74 (Abcam, Cambridge, MA), rabbit anti-CD59 (Abcam, Cambridge, MA), rabbit anti-CD70 (LifeSpan Biosciences, Seattle, WA), goat anti-TGFBI (LifeSpan Biosciences, Seattle, WA), and rabbit anti-TGM2 (Cell Signaling, Boston, MA), respectively, at the optimal dilutions according to the manufacturer's instructions. For IHC double staining of CD74 with NFkB or CD3, rabbit anti-CD74 was mixed with mouse anti-NFkB p65 (Abcam, Cambridge, MA) and mouse anti-CD3 (Cell Signaling Technology, Danvers, MA), respectively, at the optimal dilutions. The antibody mixture was then incubated with the TMAs overnight at 4 °C. Then DyLight 549 anti-mouse IgG (H+L) and DyLight 488 anti-rabbit IgG (H+L) (Vector laboratories, Burlingame, CA), or Alexa Fluor 488 anti-goat IgG (H+L) (Life Technologies, Grand Island, NY) were diluted (1:200) and incubated with the TMAs for 1 hr at room temperature. Nuclei visualization was explored by DAPI counterstaining. Between each step, there were three washes with PBST for 10 min each. Finally, the TMAs were dehydrated in alcohol and coverslipped using a CC/Mount™ permanent mounting medium (Sigma).

Results and Discussion

Pancreatic adenocarcinoma (PDAC) is characterized by a highly malignant neoplasm due to the lack of early diagnosis and extensive local invasion.¹⁸ Putative pancreatic CSCs, with a CD44⁺CD24⁺ESA⁺ phenotype, possess the ability to initiate, sustain and propagate tumor growth.¹⁰ However, the limited number of CD44⁺CD24⁺ESA⁺ cells (0.2–0.8% of pancreatic cancer cells)¹⁰ impedes the proteome study of this subpopulation. We therefore investigated the percentage of CD44⁺CD24⁺ cells by double immunostaining of CD44 and CD24 on a variety of clinical PDAC specimens and normal pancreas (Supplemental Information). The overlap of CD44 and CD24 was observed on less than 1% of pancreatic cancer cells (Supplementary Figure S1), which was consistent with the result of flow cytometry analysis (0.5–2%)¹⁰. Consistent with a previous IHC study¹⁹, the abundant expression of CD44 was detected in both normal pancreas and PDAC specimens (Supplementary Figure S1). In contrast, CD24 is negative in normal pancreas while highly overexpressed in PDAC.²⁰ Taken together with the highly tumorigenic potential of CD24 as a single marker, we investigated the proteome analysis of CD24⁺ cells procured from clinical PDAC specimens.

In clinical cases, more than 80% of PDAC patients present with a locally advanced tumor or distant metastasis at the time of diagnosis, and 74% die within the first year of diagnosis.¹ In contrast to the poor outcome of advanced pancreatic cancer, patients with an early-stage cancer have a significantly better prognosis, approaching a 5-year survival rate of 30–60%

following surgical resection.¹ Studies on the early-stage lesions provide an attractive opportunity for the development of early detection and therapeutic intervention for this lethal disease. For this purpose, CD24⁺ and CD24⁻ cells were isolated from patient-matched early-stage PDACs and their adjacent normal tissue specimens, respectively, followed by proteomic analysis.

Proteomics Workflow

We have developed a method of analyzing pure cell subpopulations isolated from frozen tissue sections combining immunohistochemistry, laser microdissection, and shotgun proteome analysis. The proteomic workflow used in this study is summarized in Figure 1. Patient-matched fresh frozen pancreatic adenocarcinoma (PDAC, n = 3) and adjacent normal tissue (ANT, n = 3) specimens were used to obtain CD24⁺ and CD24⁻ cells, respectively. iLCM was performed as an effective technique to harvest pure cell populations from the complex tissue sections. Protein extraction and digestion were accomplished using the FASP method. After tryptic digestion, peptides were separated by reverse-phase chromatography and analyzed by tandem mass spectrometry. Because of the low number of cells procured from LCM, proteomic analysis was conducted using label-free quantitation, minimizing potential sample losses during the labeling procedure. Finally, the differentially expressed proteins were validated by immunohistochemical analysis of tissue microarrays, including 8 normal pancreas tissues and 40 cases of pancreatic adenocarcinomas.

Microdissection of CD24⁺ and CD24⁻ Cells from Frozen Tissues

CD24⁺ and CD24⁻ cells were procured from PDAC and patient-matched ANT tissues, respectively. Figure 2 shows pre- and post-microdissected tissue images and the procured cells on the cap. Approximately 8.0 mm² surface area, which is equal to 40 nL tissue, was microdissected per specimen. We estimate that ~ 15 000 cells were procured from each specimen, however the estimate is imprecise since the sizes of cells in tissues are heterogeneous.

FASP for Analysis of Low Sample Amount

In shotgun proteomics, the sample preparation process includes cell lysis, protein extraction and digestion, and injection of peptides into the LC-MS/MS system for analysis. Any of these steps can potentially result in loss of proteins or peptides, which is subsequently detrimental to the proteome coverage, especially when handling a small number of cells procured from tissue sections. The filter-aided sample preparation method (FASP) has been demonstrated to allow efficient analysis of lysates from low numbers of cells.¹⁴ The sample is first solubilized with 4% SDS, and then an ultrafiltration device is used as a 'proteomic reactor' for detergent removal, buffer exchange and protein digestion, which can improve the peptide yield in the low to submicrogram range.¹⁴ In this study, 40 nL tissues were dissected from each specimen, followed by protein extraction and tryptic digestion using the FASP method, and then subject to tandem MS analysis in triplicate.

LC-MS/MS analysis

Peptides obtained from CD24⁺ PDAC or CD24⁻ ANT cells were separated by reverse-phase chromatography and analyzed by LC-MS/MS using an Orbitrap Elite mass spectrometer. It has been demonstrated that antibodies used in the immunostaining process of iLCM do not affect protein identification in proteomic analysis.²¹ Rigorous washes with PBS after incubation with antibodies remove most animal proteins from the tissue sections. The ratio between antibodies and samples is extremely low, and the minimal antibody peptides should not cause ion suppression for peptide analysis of real samples. As an example, a

representative base peak chromatogram of CD24⁺ PDAC cells and a typical MS/MS spectrum are shown in Supplementary Figure S2.

For each single run, around 3200–4700 unique peptides, corresponding to 900–1300 proteins, were identified with a peptide FDR < 0.01. The number of proteins, spectral counts and unique peptides identified in each individual run are listed in the supplementary Table S4. The comparison of three technical replicates for each sample showed that approximately 82% of proteins and 80% of unique peptides were found in at least two replicates, with an overlap of over 65% of proteins and 68% of unique peptides identified among triplicates (data not shown). The comparison across the three biological replicates showed that over 53% and 66% of proteins were identified in at least two biological replicates of CD24⁺ PDAC and CD24[−] ANT cells, respectively (Figure 3). A total of 2665 proteins were identified across the six specimens, with 847 of these proteins unique to CD24⁺ PDAC cells, 555 unique to CD24[−] ANT cells and 1263 proteins common to both cell populations (Figure 4A). The detailed information for each protein, including accession number, gene, and peptide count, and the sum of spectral counts for each individual LC-MS/MS run, are listed in Supplementary Table S2. CD24 was not observed in the mass spectra, probably due to the low tryptic digestion efficiency of this small protein, which is composed mostly of carbohydrate, with a protein core of only 31–35 amino acids.²²

We further performed Gene Ontology (GO) analysis of CD24⁺ PDAC and CD24[−] ANT cells using the FatioGO (Fast Assignment and Transference of Information using Gene Ontology) tool, a pioneering web-based tool for finding significant differences in the distribution of GO terms between groups of genes.²³ Cellular component analysis revealed a high content of membrane proteins (29–30%) and plasma membrane proteins (8–10%) (Figure 4B). Moreover, a total of 371 (17.6%) and 335 (18.4%) proteins were annotated as ‘integral to membrane’ in PDAC and ANT samples, respectively. These data are consistent with the improvement of FASP in coverage of the membrane and membrane associated proteome.²⁴ FatioGO annotation showed that proteins with a significantly increased percentage in CD24⁺ PDAC cells are primarily located in the extracellular region, cell surface, and plasma membrane. The comparison of GO annotation for biological process discovered significant alterations between CD24⁺ PDAC and CD24[−] ANT cells (Figure 4C). The percentage of proteins involved in transcription, signal transduction and cell communication increased by 20–25% in CD24⁺ PDAC cells versus CD24[−] ANT cells, paralleled by a decrease in the percentage of proteins annotated for metabolic processes (Figure 4C). These results suggest a potential signaling pathway that appears to be significantly altered in CD24⁺ cells in pancreatic adenocarcinoma.

Determination of Differentially Expressed Proteins

Proteins identified from patient-matched CD24⁺ PDAC and CD24[−] ANT specimens were quantified using a spectral counting method, which has been commonly used for measuring protein abundance in label-free shotgun proteomics.¹⁶ To achieve a quantification, we first captured CD24⁺ and CD24[−] cells with the same volume, 40 nL, from pancreatic adenocarcinoma and adjacent normal tissues, respectively. Then, the spectral counts for each protein were normalized by the total spectral counts identified in each LC-MS/MS run²⁵ to reduce the variance between samples and replicates. The fold-change was calculated as the ratio of the average normalized spectral count for the target protein between CD24⁺ PDAC and CD24[−] ANT cells. The *p*-value was generated by Student’s *t*-test across the six biological specimens (3 technical replicates each specimen), reflecting the statistical significance of the difference in protein expression. The cutoff for differential expression was set at a 2-fold change between CD24⁺ PDAC and CD24[−] ANT cells with *p*-value < 0.01.

There are 375 proteins identified as commonly differentially expressed between CD24⁺ PDAC and CD24⁻ ANT in the three patient-matched sample pairs. Among these common proteins, were 148 proteins overexpressed and 227 underexpressed in all the three PDAC specimens. The accession number, gene name, the number of peptides, normalized spectral count ratio of CD24⁺ PDAC to CD24⁻ ANT cells and *p*-value of these proteins are outlined in the Supplementary Table S5. With the isolation of CD24⁺ and CD24⁻ cells from PDAC and adjacent normal tissues, respectively, we achieved an in-depth proteome profiling with 375 differentially expressed proteins identified, however, many low abundant biomarkers remain unidentified at this stage by using the iLCM-LC-MS/MS approach.

Of the 148 proteins significantly overexpressed in CD24⁺ PDAC cells, 27 were reported previously as significantly upregulated in pancreatic cancer tissues with validation evidence in the scientific literature, indicating a good correlation between our data and previous publications. The Supplementary Table S6 lists all 27 proteins, spectral counts, the ratio of CD24⁺ PDAC to CD24⁻ ANT, and references of validation evidence. These proteins include: Anterior gradient protein 2 (AGR2), whose elevation occurs in early pancreatic cancer progression;²⁶ TGF-beta-induced protein ig-h3 (TGFBI), which is involved in TGF- β signaling;³ Gelsolin (GSN)²⁷ and Periostin (POSTN)²⁸, which are involved in the modulation of cell motility and promote the invasiveness of pancreatic tumor cells; Stratifin/14-3-3 sigma (SFN), which is up-regulated in PDAC²⁹ and lymph node metastases⁸; and Annexin A1 (ANXA1), which is correlated with poor prognosis of PDAC³⁰. The retrieval of these known potential markers showed the potential of this proteomic approach to identify markers for pancreatic cancer.

Signaling Pathways

To identify biological and molecular networks underlying CD24⁺ cells in pancreatic adenocarcinoma, a list of 375 commonly differentially expressed proteins between CD24⁺ PDAC and CD24⁻ ANT cells in the three patient-matched sample pairs was uploaded into the pathway analysis tool IPA (Ingenuity Systems®). The most variant and relevant canonical signaling pathways enriched from the 375 differentially expressed proteins were generated by IPA and ranked by significance. Figure 5 shows the canonical signaling pathways with a threshold of *p*-value < 0.01. The length of the bars indicates the significance of the signaling pathways of which the differentially expressed proteins are involved.

These enriched pathways can be grouped into three major categories: dysregulation of cell proliferation, resistance to apoptosis, and modulation of cell migration and invasion. The most prominent signaling pathways, EIF2 and Regulation of EIF4 Signaling, regulate the initiation of protein synthesis (translation)³¹ and play a critical role in cell cycle progression.³² Aryl Hydrocarbon Receptor (AhR) Signaling is also involved in cell cycle regulation, and AhR antagonism has been reported to promote the self-renewal and ex-vivo expansion of hematopoietic stem cells.³³ mTOR Signaling impacts many major cellular processes, giving it an outsized role in regulating cell growth and proliferation.³⁴ ILK Signaling plays an important role in regulating apoptosis, with consequent defects in apoptosis and resistance to cell death, which are the major causes of tumorigenesis.³⁵ Pathways mapped to modulate cell motility and surface remodeling and thus regulate cell migration and invasion include Epithelial Adherens Junction Signaling, Actin Cytoskeleton Signaling, and Regulation of Actin-based Motility by Rho³⁶. These enriched signaling pathways possess the oncogenic properties of cancer stem cells, which are consistent with the role of CD24 in stimulating tumor cell proliferation and promoting cell migration and invasion¹².

Connectivity Network Analysis

To further reveal the functional relevance of these significantly deregulated proteins, we constructed connectivity networks by IPA. The top three networks which have the highest scores are relevant to Cellular Movement, Cell-to-Cell Signaling, and Cellular Growth and Proliferation. Sixty-eight proteins are involved in these networks and are listed in Table 1 with the ratio of CD24⁺ PDAC to CD24⁻ ANT in the three patient-matched sample pairs. The subcellular location and molecular function were annotated based on the UniProt database. The networks consisting of these signature proteins are shown in Figure 6 and Supplementary Figure S3. In the following text, we discuss some of the key proteins involved in these networks.

Proteins Promoting Cancer Cell Migration and Invasion—Interestingly, many of the overexpressed proteins involved in these networks have molecular functions as extracellular matrix (ECM) structural constituents, structural constituents of cytoskeleton, or cell surface binding proteins, such as Prolargin, Fibulin-2, Fibronectin, Collagen type I, Talin-1, Spectrin and Fibrinogens (Table 1).³⁷ Tumor cells interact with the ECM during invasion and metastasis, which involves adherence of the tumor cells to the ECM, degradation of matrix components, and movement of the cell body.³⁸ It has been reported that the aberrant expression of ECM proteins promotes the affinity of pancreatic cancer cells for the tumor invasion.³⁹ Moreover, TSPAN8, also known as C0-029 in humans, was highly upregulated in CD24⁺ PDAC cells. TSPAN8 belongs to a large family of integral membrane proteins, and its overexpression strongly correlates with increased tumor cell motility in pancreatic adenocarcinoma.⁴⁰ These results were in good agreement with the high invasion ability of tumor cells expressing CD24.¹²

Proteins Mediating Immune Escape—Proteins which have been reported to play key roles in mediation of immune escape in human tumors include CD59, CD70, and CD74. These proteins, with molecular functions of cell surface receptor, cytokine receptor, and signal transducer activity respectively, were only expressed in CD24⁺ PDAC cells (Table 1). It should be noted that CD70 and CD74 were identified, for the first time, as highly upregulated in PDAC using the proteomics approach. CD59, also called Protectin, is a membrane-bound complement regulatory protein (CRP) that inhibits the cytolytic activity of complement by binding to C8 and C9, thereby blocking the assembly of the membrane attack complex.⁴¹ The CRPs are overexpressed on the cell surface of human malignancies, allowing tumor cells to escape immune surveillance.⁴² CD70, a member of the tumor necrosis factor (TNF) receptor family⁴³, is a cell surface protein expressed in antigen-activated T and B lymphocytes, whose interaction with CD27 regulates B- and T-cell functions.⁴⁴ CD70 is highly overexpressed in various solid tumors, where its up-regulation can induce lymphocyte apoptosis through interaction with its receptor CD27, leading to immune suppression.^{45–46} CD74 is a transmembrane glycoprotein that associates with the major histocompatibility complex (MHC) class II and participates in several key processes of the immune system, including antigen presentation, B-cell differentiation and inflammatory signaling.⁴⁷ Aberrant expression of CD74 has been reported in some malignant tumors, where it interacts with HLA-DR molecules and this interaction prevents binding of HLA-DR molecules to endogenous peptides and blocks the host immune response to tumor cells.⁴⁸ Together, these proteins may facilitate CD24⁺ PDAC cells, a highly tumorigenic subpopulation in PDAC, to escape immune surveillance and promote cell survival.

Proteins Associated with Tumor Progression—Several molecules were identified with key involvement in tumor progression, including 14-3-3 sigma and PDCD4. 14-3-3 sigma (SFN) was highly upregulated in CD24⁺ PDAC cells (Table 1). It belongs to the 14-

3-3 protein family, which plays a role in the modulation of diverse signal transduction pathways that are implicated in cancer development.⁴⁹ Interestingly, SFN is imported to generate two networks by IPA (Figure 6A and S3), suggesting a potential involvement in both cellular movement and cellular growth and proliferation. Programmed cell death protein 4 (PDCD4), known as a tumor suppressor, is lost in certain aggressive malignant diseases,⁵⁰ and was found significantly downregulated in CD24⁺ PDAC cells. There is accumulating evidence that PDCD4 plays an important role in the control of tumorigenesis and its degradation has important consequences in cell proliferation and neoplastic transformation.⁵¹ A study revealed that PDCD4 is targeted for degradation during tumor promotion,⁵² therefore, inhibiting signaling to PDCD4 degradation may prove a valid strategy for cancer therapies.

Immunohistochemistry Validation

Four proteins of interest, CD59, CD70, CD74 and TGFBI, were selected for verification of their differential expression in CD24⁺ PDAC compared to CD24⁻ ANT cells. The selection of these proteins was based on the MS results (unique to CD24⁺ PDAC or highly overexpressed in CD24⁺ PDAC) and their important roles in the networks of Cell-to-Cell Signaling and Cellular Growth and Proliferation. The overlap of these candidate proteins with CD24 was investigated by double fluorescent immunohistochemistry on tissue microarrays, which contains 40 pancreatic adenocarcinomas with clinical stages and pathology grades and 8 normal pancreas specimens. The distribution of protein expression was evaluated as the percentage of stained cells (0 = no staining; 1 = < 10%; 2 = 10–50%; 3 = 51–80%; 4 = > 80%) and the staining intensity (0 = no staining; 1 = weak; 2 = moderate; 3 = strong), according to the previous study⁵³. When the multiplication product of the two scores was greater than 2, it was considered positively stained. The immunohistochemical analysis of the four candidates and CD24 in normal and PDAC tissues are summarized in Table S7. As shown in Table S7 and Figure 7, all four proteins showed significantly higher expression in a large group of clinical PDACs, whereas all were negative in normal pancreas samples.

Consistent with a previous IHC study²⁰, CD24 showed sparse expression in acinar cells of normal pancreas tissues, with less than 1% CD24⁺ cells, which was considered negative as defined above. In PDAC, CD24 expression was observed in 85% (34/40) of cases, with strong or moderate staining on both membrane and cytoplasm of tumor cells (Figure 7). It exhibited a significant correlation with high-grade tumors as reported previously⁵³, predominantly present on the apical membrane of malignant ducts and vessels.

CD59 Immunostaining—The staining with CD59 exhibited strong membrane positivity in PDAC, which was observed in 45% (15/40) of PDAC samples (Table S7). However, it was negative in normal pancreas (Figure 7). Strong CD59 staining was present on the apical membrane surface of tumor cells in early-stage PDACs, while a varying expression was observed in late-stage PDACs with the positivity primarily in malignant tumor cells and ducts. Double fluorescent staining showed the CD59 was mostly coexpressed with CD24 in PDAC (shown as *yellow* or *orange* in Figure 7). CD59, as one of the complement regulatory proteins (CRPs), can inhibit complement lysis and prevent formation of the membrane attack complex, and its overexpression on the cell surface allows tumor cells to escape immune surveillance.⁴² The co-expression of CD59 with CD24 suggested that it may facilitate pancreatic CD24⁺ cancer cells to escape immune attack.

CD70 Immunostaining—The staining of CD70 showed strong cytoplasmic and membrane positivities in tumor cells, which was observed in 25 of 50 PDAC specimens (50%); however, it was negative in normal pancreas (Table S7). Both CD70 and CD24

showed a minimal expression in normal pancreas, with less than 1% CD70⁺ and CD24⁺ cells, which were considered negative as described above. Interestingly, CD70 was exclusively coexpressed with CD24 in normal pancreas. In early stages of PDAC, strong membrane and cytoplasmic CD70 staining was co-localized with CD24 (shown as *yellow* in Figure 7). However, it exhibited weak membrane expression in malignant ducts of late-stage tumors (Figure 7). The comparison showed that CD70 overexpression was correlated with early-stage PDAC (Table S7). There is increasing evidence that the upregulation of CD70 in solid tumors mediates apoptosis of immune effector cells and results in immune escape.⁴⁵ The significant coexpression of CD70 and CD24 in early-stage PDACs suggests that CD70 may serve as a potential marker and an attractive target, together with CD24, for tumor initiating cells of pancreatic cancer.

CD74 Immunostaining—The aberrant expression of CD74 was observed in 16 of 40 cases (40%) of PDAC, of which 12 (30%) cases showed strong CD74 staining. However, no staining was observed in normal pancreas (Table S7). Strong immunoreactivity of CD74 was generally observed in the cytoplasm and sometime along with the membranes of tumor cells. It also showed a weak expression in CD24⁺ cells in early-stage PDACs (shown as *orange* in Figure 7) and varying expression in late stage PDACs. Overall, the overlap of CD74 and CD24 was present in a small population of tumor cells. CD74 expression on malignant cells has been reported to prevent presentation of tumor antigen and, as a consequence, tumor cells are less immunogenic and can maintain their proliferation potential.⁴⁸ The important role of CD74 in inflammatory and carcinogenic processes has been outlined as is the use of CD74 as a therapeutic target in cancer.⁴⁷ Elevated expression of CD74 in PDAC suggests that it may serve as a prognostic marker for tumor progression of pancreatic cancer.⁵⁴

As shown in Figure 6B, both NFκB and CD3, which are imported as critical nodes to generate the interaction network of cell-to-cell signaling by IPA, are directly connected with CD74, suggesting the potential involvement and interactions with CD74 in this network although they were not seen in the proteomic data. We therefore performed the double fluorescent immunostaining of NFκB p65 and CD3 with CD74, respectively, on the tissue microarrays. The IHC result was shown in Supplementary Figure S4. Interestingly, both NFκB p65 and CD3 were highly coexpressed with CD74 at early stages of PDACs, showing moderate to strong cytoplasmic immunoreactivity (Figure S4B, S4C, S4E, and S4F). The expression of NFκB p65 and CD3 was decreased at late stages of PDACs (data not shown). In normal pancreas, they showed weak and sparse expression (Figure S4A and S4D), which was considered negative according to the scoring system described above. The significant coexpression of NFκB p65 and CD3 with CD74 at early stages of PDACs further confirmed their involvement in the cell signaling network and potential interactions with CD74.

TGFBI Immunostaining—Transforming growth factor-beta-induced protein ig-h3 (TGFBI) was found with an average 32-fold increase in CD24⁺ PDAC cells versus CD24-ANT cells (Table S5), which was consistent with the 32.4-fold increase in the mRNA level in pancreatic cancer tissues compared to normal tissues⁵⁵. Enhanced TGFBI expression has been confirmed in PDAC specimens.³ Here, we further investigated the overlap between TGFBI and CD24 in pancreatic cancer. IHC staining of TGFBI revealed positive expression in 30 (75%) of the 40 PDAC patients, showing a significant correlation with late stages (Table S7). In normal pancreas, TGFBI showed sparse expression, with less than 1% TGFBI⁺ cells, and was restrictedly coexpressed with CD24 in acinar cells (Figure 7). TGFBI expression was strongly present on the cytoplasm and membrane of cancer cells at early stages, with a predominant co-localization with CD24 (shown as *orange* in Figure 7). The insert also shows the apical membrane expression of TGFBI in a malignant duct at stage II. In late stages, intense TGFBI expression was abundantly observed on the membrane of

malignant ductal cells, displaying an apparent overlap with CD24. The immunohistochemistry results confirmed the significantly high expression of TGFBI in CD24⁺ PDAC cells. The significant correlation of TGFBI expression with late stages of PDACs suggested that it may serve as a promising marker in the prediction of prognosis for pancreatic adenocarcinoma.

Conclusion

This work represents a comprehensive proteome analysis of a highly tumorigenic subpopulation within pancreatic adenocarcinoma (PDAC), CD24⁺ cells, which were derived from fresh frozen human PDAC specimens at early stages, and a comparison with CD24⁻ cells procured from patient-matched adjacent normal tissues (ANTs). In total, 2665 proteins were identified, of which 375 proteins were significantly differentially expressed in all three patients by at least a 2-fold change in CD24⁺ PDAC compared to CD24⁻ ANT cells. Among them, 27 known candidate markers were retrieved, demonstrating the potential of this approach for pancreatic cancer biomarker discovery. Ingenuity Pathway analysis (IPA) of the differentially expressed proteins revealed 68 signature proteins involved in the high-scoring biological networks which are relevant to cellular movement, cell-to-cell signaling and cellular growth and proliferation. The major groups of these proteins are involved in promoting tumor cell migration and invasion, immune escape and tumor progression, which may contribute to the high tumorigenic potential¹⁰ and improved invasion ability¹² of pancreatic CD24⁺ adenocarcinoma cells. The results presented here are not only complementary to the current pancreatic proteome, but may provide an effective approach to uncover novel markers and important events in pancreatic tumorigenesis.

Furthermore, immunohistochemistry analysis of four selected candidate proteins, CD59, CD70, CD74 and TGFBI, verified that they are highly overexpressed in a large group of clinical PDAC, but are all negative in normal pancreas. The aberrant overexpression of CD59, CD70, and CD74 in human malignancies has been reported to play essential roles in mediating immune responses and allowing tumor cells to escape immune surveillance.^{42, 45, 47} The coexpression of these proteins with CD24 in PDAC implicates that they may facilitate CD24⁺ PDAC cells, a highly tumorigenic subpopulation within PDAC, to escape immune attack and promote cell survival. In addition, the predominant co-localization of CD70 and CD24 in early-stage PDAC suggests that this cell population could be of particular interest when attempting to target tumor-initiating cells and develop novel therapeutics for pancreatic cancer. The persistent coexpression of TGFBI with CD24 in all stages, particularly exhibiting a significant association with late stages, suggests that it may serve as a promising prognostic marker for pancreatic cancer. Preliminary results are promising and warrant further studies on these key proteins as markers and potential therapeutic targets for pancreatic adenocarcinoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported under the National Institutes of Health Grant R01 49500 (D.M.L.) and the National Cancer Institute Grant 1R01CA154455 (D.M.L.). We thank Dr. Philip Gafken of the Fred Hutchinson Cancer Research Center for running the LC-MS/MS analyses on the Orbitrap Elite mass spectrometer. We thank Dr. Andy Lo (University of Michigan) for his helpful suggestions on the manuscript.

References

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. *CA Cancer J Clin.* 2009; 59(4):225–249. [PubMed: 19474385]
2. Lowery MA, O'Reilly EM. Pancreatic cancer: the role of molecular markers in diagnosis and management. *Clin Adv Hematol Oncol.* 2011; 9(12):900–908. [PubMed: 22252658]
3. Turtoi A, Musmeci D, Wang Y, Dumont B, Somja J, Bevilacqua G, De Pauw E, Delvenne P, Castronovo V. Identification of novel accessible proteins bearing diagnostic and therapeutic potential in human pancreatic ductal adenocarcinoma. *J Proteome Res.* 2011; 10(9):4302–4313. [PubMed: 21755970]
4. Mahadevan D, Von Hoff DD. Tumor-stroma interactions in pancreatic ductal adenocarcinoma. *Mol Cancer Ther.* 2007; 6(4):1186–1197. [PubMed: 17406031]
5. Johann DJ, Rodriguez-Canales J, Mukherjee S, Prieto DA, Hanson JC, Emmert-Buck M, Blonder J. Approaching solid tumor heterogeneity on a cellular basis by tissue proteomics using laser capture microdissection and biological mass spectrometry. *J Proteome Res.* 2009; 8(5):2310–2318. [PubMed: 19284784]
6. Espina V, Heiby M, Pierobon M, Liotta LA. Laser capture microdissection technology. *Expert Rev Mol Diagn.* 2007; 7(5):647–657. [PubMed: 17892370]
7. Shekouh AR, Thompson CC, Prime W, Campbell F, Hamlett J, Herrington CS, Lemoine NR, Crnogorac-Jurcevic T, Buechler MW, Friess H, Neoptolemos JP, Pennington SR, Costello E. Application of laser capture microdissection combined with two-dimensional electrophoresis for the discovery of differentially regulated proteins in pancreatic ductal adenocarcinoma. *Proteomics.* 2003; 3(10):1988–2001. [PubMed: 14625861]
8. Naidoo K, Jones R, Dmitrovic B, Wijesuriya N, Kocher H, Hart IR, Crnogorac-Jurcevic T. Proteome of formalin-fixed paraffin-embedded pancreatic ductal adenocarcinoma and lymph node metastases. *J Pathol.* 2012; 226(5):756–763. [PubMed: 22081483]
9. Liu Y, Wu J, Yan G, Hou R, Zhuang D, Chen L, Pang Q, Zhu J. Proteomic analysis of prolactinoma cells by immuno-laser capture microdissection combined with online two-dimensional nano-scale liquid chromatography/mass spectrometry. *Proteome Sci.* 2010; 8:2. [PubMed: 20205839]
10. Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, Wicha M, Clarke MF, Simeone DM. Identification of pancreatic cancer stem cells. *Cancer Res.* 2007; 67(3):1030–1037. [PubMed: 17283135]
11. Kitaura Y, Chikazawa N, Tasaka T, Nakano K, Tanaka M, Onishi H, Katano M. Transforming growth factor beta1 contributes to the invasiveness of pancreatic ductal adenocarcinoma cells through the regulation of CD24 expression. *Pancreas.* 2011; 40(7):1034–1042. [PubMed: 21697762]
12. Baumann P, Cremers N, Kroese F, Orend G, Chiquet-Ehrismann R, Uede T, Yagita H, Sleeman JP. CD24 expression causes the acquisition of multiple cellular properties associated with tumor growth and metastasis. *Cancer Res.* 2005; 65(23):10783–10793. [PubMed: 16322224]
13. Ikenaga N, Ohuchida K, Mizumoto K, Yu J, Kayashima T, Hayashi A, Nakata K, Tanaka M. Characterization of CD24 expression in intraductal papillary mucinous neoplasms and ductal carcinoma of the pancreas. *Hum Pathol.* 2010; 41(10):1466–1474. [PubMed: 20619441]
14. Wisniewski JR, Ostasiewicz P, Mann M. High recovery FASP applied to the proteomic analysis of microdissected formalin fixed paraffin embedded cancer tissues retrieves known colon cancer markers. *J Proteome Res.* 2011; 10(7):3040–3049. [PubMed: 21526778]
15. Michalski A, Damoc E, Lange O, Denisov E, Nolting D, Muller M, Viner R, Schwartz J, Remes P, Belford M, Dunyach JJ, Cox J, Horning S, Mann M, Makarov A. Ultra high resolution linear ion trap Orbitrap mass spectrometer (Orbitrap Elite) facilitates top down LC MS/MS and versatile peptide fragmentation modes. *Mol Cell Proteomics.* 2012; 11(3):O111 013698. [PubMed: 22159718]
16. Liu H, Sadygov RG, Yates JR 3rd. A model for random sampling and estimation of relative protein abundance in shotgun proteomics. *Anal Chem.* 2004; 76(14):4193–4201. [PubMed: 15253663]
17. Colinge J, Chiappe D, Lagache S, Moniatte M, Bougueleret L. Differential proteomics via probabilistic peptide identification scores. *Anal Chem.* 2005; 77(2):596–606. [PubMed: 15649059]

18. Philip PA, Mooney M, Jaffe D, Eckhardt G, Moore M, Meropol N, Emens L, O'Reilly E, Korc M, Ellis L, Benedetti J, Rothenberg M, Willett C, Tempero M, Lowy A, Abbruzzese J, Simeone D, Hingorani S, Berlin J, Tepper J. Consensus report of the national cancer institute clinical trials planning meeting on pancreas cancer treatment. *J Clin Oncol*. 2009; 27(33):5660–5669. [PubMed: 19858397]
19. Immervoll H, Hoem D, Steffensen OJ, Miletic H, Molven A. Visualization of CD44 and CD133 in normal pancreas and pancreatic ductal adenocarcinomas: non-overlapping membrane expression in cell populations positive for both markers. *J Histochem Cytochem*. 2011; 59(4):441–455. [PubMed: 21411814]
20. Zhu J, He J, Liu Y, Simeone DM, Lubman DM. Identification of glycoprotein markers for pancreatic cancer CD24+CD44+ stem-like cells using nano-LC-MS/MS and tissue microarray. *J Proteome Res*. 2012; 11(4):2272–2281. [PubMed: 22335271]
21. Mouldous L, Hunt S, Harcourt R, Harry JL, Williams KL, Gutstein HB. Proteomic analysis of immunostained, laser-capture microdissected brain samples. *Electrophoresis*. 2003; 24(1–2):296–302. [PubMed: 12652601]
22. Kay R, Rosten PM, Humphries RK. CD24, a signal transducer modulating B cell activation responses, is a very short peptide with a glycosyl phosphatidylinositol membrane anchor. *J Immunol*. 1991; 147(4):1412–1416. [PubMed: 1831224]
23. Al-Shahrour F, Minguez P, Tarraga J, Medina I, Alloza E, Montaner D, Dopazo J. FatiGO +: a functional profiling tool for genomic data. Integration of functional annotation, regulatory motifs and interaction data with microarray experiments. *Nucleic Acids Res*. 2007; 35(Web Server issue):W91–96. [PubMed: 17478504]
24. Wisniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. *Nat Methods*. 2009; 6(5):359–362. [PubMed: 19377485]
25. Dong MQ, Venable JD, Au N, Xu T, Park SK, Cociorva D, Johnson JR, Dillin A, Yates JR 3rd. Quantitative mass spectrometry identifies insulin signaling targets in *C. elegans*. *Science*. 2007; 317(5838):660–663. [PubMed: 17673661]
26. Chen R, Pan S, Duan X, Nelson BH, Sahota RA, de Rham S, Kozarek RA, McIntosh M, Brentnall TA. Elevated level of anterior gradient-2 in pancreatic juice from patients with pre-malignant pancreatic neoplasia. *Mol Cancer*. 2010; 9:149. [PubMed: 20550709]
27. Thompson CC, Ashcroft FJ, Patel S, Saraga G, Vimalachandran D, Prime W, Campbell F, Dodson A, Jenkins RE, Lemoine NR, Crnogorac-Jurcevic T, Yin HL, Costello E. Pancreatic cancer cells overexpress gelsolin family-capping proteins, which contribute to their cell motility. *Gut*. 2007; 56(1):95–106. [PubMed: 16847067]
28. Baril P, Gangeswaran R, Mahon PC, Caulee K, Kocher HM, Harada T, Zhu M, Kalthoff H, Crnogorac-Jurcevic T, Lemoine NR. Periostin promotes invasiveness and resistance of pancreatic cancer cells to hypoxia-induced cell death: role of the beta4 integrin and the PI3k pathway. *Oncogene*. 2007; 26(14):2082–2094. [PubMed: 17043657]
29. Sitek B, Sipos B, Alkatout I, Poschmann G, Stephan C, Schulenburg T, Marcus K, Luttges J, Dittert DD, Baretton G, Schmiegeler W, Hahn SA, Kloppel G, Meyer HE, Stuhler K. Analysis of the pancreatic tumor progression by a quantitative proteomic approach and immunohistochemical validation. *J Proteome Res*. 2009; 8(4):1647–1656. [PubMed: 19714807]
30. Bai XF, Ni XG, Zhao P, Liu SM, Wang HX, Guo B, Zhou LP, Liu F, Zhang JS, Wang K, Xie YQ, Shao YF, Zhao XH. Overexpression of annexin 1 in pancreatic cancer and its clinical significance. *World J Gastroenterol*. 2004; 10(10):1466–1470. [PubMed: 15133855]
31. Proud CG. eIF2 and the control of cell physiology. *Semin Cell Dev Biol*. 2005; 16(1):3–12. [PubMed: 15659334]
32. Clemens MJ. Translational regulation in cell stress and apoptosis. Roles of the eIF4E binding proteins. *J Cell Mol Med*. 2001; 5(3):221–239. [PubMed: 12067482]
33. Boitano AE, Wang J, Romeo R, Bouchez LC, Parker AE, Sutton SE, Walker JR, Flaveny CA, Perdew GH, Denison MS, Schultz PG, Cooke MP. Aryl hydrocarbon receptor antagonists promote the expansion of human hematopoietic stem cells. *Science*. 2010; 329(5997):1345–1348. [PubMed: 20688981]

34. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell*. 2012; 149(2):274–293. [PubMed: 22500797]
35. Attwell S, Roskelley C, Dedhar S. The integrin-linked kinase (ILK) suppresses anoikis. *Oncogene*. 2000; 19(33):3811–3815. [PubMed: 10949937]
36. Azios NG, Krishnamoorthy L, Harris M, Cubano LA, Cammer M, Dharmawardhane SF. Estrogen and resveratrol regulate Rac and Cdc42 signaling to the actin cytoskeleton of metastatic breast cancer cells. *Neoplasia*. 2007; 9(2):147–158. [PubMed: 17356711]
37. Karsdal MA, Nielsen MJ, Sand JM, Henriksen K, Genovese F, Bay-Jensen AC, Victoria S, Adamkewicz JI, Christiansen C, Leeming DJ. Extracellular Matrix Remodeling: The Common Denominator in Connective Tissue Diseases Possibilities for Evaluation and Current Understanding of the Matrix as More Than a Passive Architecture, But a Key Player in Tissue Failure. *Assay Drug Dev Technol*. 2012
38. Crowe DL, Shuler CF. Regulation of tumor cell invasion by extracellular matrix. *Histol Histopathol*. 1999; 14(2):665–671. [PubMed: 10212827]
39. Ryschich E, Khamidjanov A, Kerkadze V, Buchler MW, Zoller M, Schmidt J. Promotion of tumor cell migration by extracellular matrix proteins in human pancreatic cancer. *Pancreas*. 2009; 38(7):804–810. [PubMed: 19893454]
40. Gesierich S, Paret C, Hildebrand D, Weitz J, Zraggen K, Schmitz-Winnenthal FH, Horejsi V, Yoshie O, Herlyn D, Ashman LK, Zoller M. Colocalization of the tetraspanins, CO-029 and CD151, with integrins in human pancreatic adenocarcinoma: impact on cell motility. *Clin Cancer Res*. 2005; 11(8):2840–2852. [PubMed: 15837731]
41. Meri S, Morgan BP, Davies A, Daniels RH, Olavesen MG, Waldmann H, Lachmann PJ. Human protectin (CD59): an 18,000–20,000 MW complement lysis restricting factor, inhibits C5b-8 catalysed insertion of C9 into lipid bilayers. *Immunology*. 1990; 71(1):1–9. [PubMed: 1698710]
42. Schmitt CA, Schwaeble W, Wittig BM, Meyer zum Buschenfelde KH, Dippold WG. Expression and regulation by interferon-gamma of the membrane-bound complement regulators CD46 (MCP): CD55 (DAF) and CD59 in gastrointestinal tumours. *Eur J Cancer*. 1999; 35(1):117–124. [PubMed: 10211099]
43. Goodwin RG, Alderson MR, Smith CA, Armitage RJ, VandenBos T, Jerzy R, Tough TW, Schoenborn MA, Davis-Smith T, Hennen K, et al. Molecular and biological characterization of a ligand for CD27 defines a new family of cytokines with homology to tumor necrosis factor. *Cell*. 1993; 73(3):447–456. [PubMed: 8387892]
44. Arens R, Tesselaar K, Baars PA, van Schijndel GM, Hendriks J, Pals ST, Krimpenfort P, Borst J, van Oers MH, van Lier RA. Constitutive CD27/CD70 interaction induces expansion of effector-type T cells and results in IFN γ -mediated B cell depletion. *Immunity*. 2001; 15(5):801–812. [PubMed: 11728341]
45. Wischhusen J, Jung G, Radovanovic I, Beier C, Steinbach JP, Rimner A, Huang H, Schulz JB, Ohgaki H, Aguzzi A, Rammensee HG, Weller M. Identification of CD70-mediated apoptosis of immune effector cells as a novel immune escape pathway of human glioblastoma. *Cancer Res*. 2002; 62(9):2592–2599. [PubMed: 11980654]
46. Diegmann J, Junker K, Loncarevic IF, Michel S, Schimmel B, von Eggeling F. Immune escape for renal cell carcinoma: CD70 mediates apoptosis in lymphocytes. *Neoplasia*. 2006; 8(11):933–938. [PubMed: 17132225]
47. Borghese F, Clanchy FI. CD74: an emerging opportunity as a therapeutic target in cancer and autoimmune disease. *Expert Opin Ther Targets*. 2011; 15(3):237–251. [PubMed: 21208136]
48. Ishigami S, Natsugoe S, Tokuda K, Nakajo A, Iwashige H, Aridome K, Hokita S, Aikou T. Invariant chain expression in gastric cancer. *Cancer Lett*. 2001; 168(1):87–91. [PubMed: 11368882]
49. Obsilova V, Silhan J, Boura E, Teisinger J, Obsil T. 14–3-3 proteins: a family of versatile molecular regulators. *Physiol Res*. 2008; 57(Suppl 3):S11–21. [PubMed: 18481918]
50. Chen Y, Knosel T, Kristiansen G, Pietas A, Garber ME, Matsuhashi S, Ozaki I, Petersen I. Loss of PDCD4 expression in human lung cancer correlates with tumour progression and prognosis. *J Pathol*. 2003; 200(5):640–646. [PubMed: 12898601]

51. Mudduluru G, Medved F, Grobholz R, Jost C, Gruber A, Leupold JH, Post S, Jansen A, Colburn NH, Allgayer H. Loss of programmed cell death 4 expression marks adenoma-carcinoma transition, correlates inversely with phosphorylated protein kinase B, and is an independent prognostic factor in resected colorectal cancer. *Cancer*. 2007; 110(8):1697–1707. [PubMed: 17849461]
52. Schmid T, Jansen AP, Baker AR, Hegamyer G, Hagan JP, Colburn NH. Translation inhibitor Pdc4 is targeted for degradation during tumor promotion. *Cancer Res*. 2008; 68(5):1254–1260. [PubMed: 18296647]
53. Jacob J, Bellach J, Grutzmann R, Alldinger I, Pilarsky C, Dietel M, Kristiansen G. Expression of CD24 in adenocarcinomas of the pancreas correlates with higher tumor grades. *Pancreatology*. 2004; 4(5):454–460. [PubMed: 15256807]
54. Nagata S, Jin YF, Yoshizato K, Tomoeda M, Song M, Iizuka N, Kitamura M, Takahashi H, Eguchi H, Ohigashi H, Ishikawa O, Tomita Y. CD74 is a novel prognostic factor for patients with pancreatic cancer receiving multimodal therapy. *Ann Surg Oncol*. 2009; 16(9):2531–2538. [PubMed: 19499276]
55. Schneider D, Kleeff J, Berberat PO, Zhu Z, Korc M, Friess H, Buchler MW. Induction and expression of betaig-h3 in pancreatic cancer cells. *Biochim Biophys Acta*. 2002; 1588(1):1–6. [PubMed: 12379307]

Figure 1. Shotgun proteomics workflow for the analysis of CD24⁺ and CD24⁻ cells isolated from frozen pancreatic adenocarcinoma (PDAC) and patient-matched adjacent normal tissue (ANT) specimens, respectively.

Figure 2. Immuno-laser capture microdissection (i-LCM) of CD24⁺ cells from frozen pancreatic adenocarcinoma tissue sections. Images of A) pre-microdissection, B) post-microdissection, and C) isolated CD24⁺ PDAC cells. A–C = $\times 100$ magnification.

Figure 3.

Comparison of protein numbers identified in three biological replicates for CD24⁺ and CD24⁻ cells microdissected from frozen pancreatic adenocarcinomas (PDAC, n = 3) and patient-matched adjacent normal tissues (ANTs, n = 3), respectively.

Figure 4.

Proteomic analyses of CD24⁺ PDAC and CD24⁻ ANT cells. (A) The overlap of proteins identified from CD24⁺ PDAC and CD24⁻ ANT cells. (B, C) Comparison of Gene Ontology annotations for cell component (B) and biological processes (C) of proteins identified in CD24⁺ PDAC (black bars) and CD24⁻ ANT (gray bars) cells. Lengths of the bars are proportionate to the percentage value. GO annotation revealed that proteins with a significantly increased percentage in CD24⁺ PDAC cells are primarily located in the extracellular region, cell surface, and plasma membrane. The comparison of biological processes discovered that the percentage of proteins involved in transcription, signal transduction and cell communication increased by 20–25% in CD24⁺ PDAC versus CD24⁻ ANT cells.

Figure 5.

Canonical signaling pathways enriched with commonly differentially expressed proteins between CD24⁺ PDAC and CD24⁻ ANT cells and ranked by significance in the IPA. A p -value threshold of 0.01 is applied. The enriched pathways can be grouped into three major categories: dysregulation of cell proliferation, resistance to apoptosis, and modulation of cell migration and invasion.

Figure 6.

The top two connectivity networks constructed by IPA: (A) Cellular Movement; (B) Cell-to-Cell Signaling. These networks only consist of differentially expressed proteins derived from the experimental data. *Red* and *green* represent over- and under-expression in CD24⁺ PDAC cells compared with CD24⁻ ANT cells, respectively. *White* indicates proteins that were not identified as differentially expressed in this study, but are relevant to the networks.

Figure 7.

Immunofluorescence double staining with CD24 (*red*) and four candidate proteins (*green*), CD59, CD70, CD74, and TGFBI respectively, in human normal pancreas and pancreatic adenocarcinoma of early (I and II) and late stages (III and IV). DAPI counterstaining was used to visualize nuclei (*blue*). *Orange* or *yellow* represent the co-localization of the candidate protein and CD24. Overall, these proteins were all negative in normal pancreas. CD70 and TGFBI showed a minimal expression in normal pancreas, with less than 1% CD70⁺ and 3% TGFBI⁺ cells, respectively, which were considered negative as described in the paper. CD59 showed strong membrane positivity in PDAC and highly coexpressed with CD24. CD70 exhibited strong membrane and cytoplasmic staining in early stages of PDACs, which was co-localized with CD24, but showed a weak expression in late stages of PDACs. CD74 presented a strong cytoplasmic positivity in PDAC, coexpressed with CD24 in a small population of CD24⁺ cells. TGFBI expression was strongly present on the cytoplasm and membrane of malignant cells at early stages of PDAC, with a predominant co-localization with CD24. In late stages, intense TGFBI expression was abundantly observed on the membrane of malignant ductal cells. Magnification 200×, scale bars = 50 μm.

Significantly differentially expressed proteins identified between CD24⁺ PDAC and CD24⁻ ANT cells, which were involved in the networks of Cellular Movement, Cell-to-Cell Signaling, and Cellular Growth and Proliferation.

Table 1

Ratio of CD24 ⁺ PDAC to CD24 ⁻ ANT									
Accession	Protein name	Gene	Peptide ^a	Sample pair			P-value	Location	Function
				1	2	3			
(A) Cellular Movement									
P61158	ARP3 actin-related protein 3 homolog	ACTR3	15	2.88	2.18	3.69	2.15E-06	Plasma Membrane	ATP binding
P04083	Annexin A1	ANXA1	78	3.00	12.1	4.32	6.10E-06	Plasma Membrane	phospholipase A2 inhibitor
P01024	Complement component 3	C3	92	4.43	NC	2.72	2.92E-08	Secreted	endopeptidase inhibitor activity
P02748	Complement component 9	C9	3	NC ^b	NC	NC	3.91E-05	Plasma Membrane/Secreted	complement activation
P06729	T-cell surface antigen CD2	CD2	3	NC	ND ^c	NC	9.66E-03	Membrane	eukaryotic cell surface binding; receptor activity
P13987	CD59 glycoprotein	CD59	2	NC	NC	ND	1.42E-02	Plasma Membrane	cell surface receptor signaling
Q99439	Calponin-2	CNN2	2	ND	NC	NC	9.98E-03	Cytoskeleton	cytoskeleton organization
Q16555	Dihydropyrimidinase-like 2	DPYSL2	12	NC	NC	NC	1.30E-09	Cytoplasm	Developmental protein
Q9Y285	Phenylalanyl-tRNA synthetase alpha chain	FARSA	4	0.07	0.26	0.45	1.71E-06	Cytoplasm	Aminoacyl-tRNA synthetase
P02675	Fibrinogen beta chain	FGB	32	14.40	NC	NC	1.06E-04	Secreted	eukaryotic cell surface binding
P02679	Fibrinogen gamma chain	FGG	16	9.37	NC	NC	2.32E-06	Secreted	eukaryotic cell surface binding
P11413	Glucose-6-phosphate 1- dehydrogenase	G6PD	2	3.32	NC	NC	1.92E-03	Cytosol	Oxidoreductase
P18283	Glutathione peroxidase 2	GPX2	3	NC	NC	NC	9.86E-03	Cytoplasm	Oxidoreductase
Q16787	Laminin subunit alpha-3	LAMA3	2	NC	NC	NC	5.84E-03	Membrane	structural molecule activity
Q13751	Laminin subunit beta-3	LAMB3	2	NC	NC	NC	7.39E-03	Membrane	structural molecule activity
P48059	LIM and senescent cell antigen- like-containing domain protein 1	LIMS1	3	ND	NC	NC	2.24E-03	Plasma Membrane	zinc ion binding
Q14764	Major vault protein	MVP	68	13.04	28.8	NC	5.70E-12	Cytoplasm/Nucleus	Ribonucleoprotein
P20591	Interferon-induced GTP- binding protein Mx1	MX1	15	NC	NC	ND	1.98E-03	Membrane	Hydrolase activity
Q53EL6	Programmed cell death protein 4	PDCD4	0	0.00 ^b	0.27	0.00	3.98E-08	Cytoplasm/Nucleus	RNA binding
P13797	Plastin-3	PLS3	13	NC	NC	NC	7.55E-08	Cytoplasm	calcium ion binding
P51888	Prolargin	PRELP	62	192.69	NC	ND	1.40E-03	Extracellular Matrix	extracellular matrix structural constituent

Ratio of CD24 ⁺ PDAC to CD24 ⁻ ANT										
Accession	Protein name	Gene	Peptide ^a	Sample pair			P-value	Location	Function	
				1	2	3				
P36955	Pigment epithelium-derived factor	SERPINF1	32	5.03	NC	ND	6.94E-03	Secreted	endopeptidase inhibitor activity	
P31947	14-3-3 protein sigma/Stratfin	SFN	34	NC	NC	NC	8.79E-08	Cytoplasm/Nucleus	protein kinase C inhibitor activity	
P42224	Signal transducer and activator of transcription 1-alpha/beta	STAT1	5	7.13	NC	NC	4.59E-03	Cytoplasm	activator; signal transducer activity	
P27105	Erythrocyte band 7 integral membrane protein	STOM	9	2.17	8.39	8.42	1.09E-04	Plasma Membrane	protein homooligomerization	
Q9Y490	Talin-1	TLN1	118	2.89	7.09	2.22	1.52E-09	Plasma Membrane	structural constituent of cytoskeleton	
P06753	Tropomyosin-3	TPM3	3	NC	NC	NC	2.49E-04	Cytoplasm	regulation of muscle contraction	
P19075	Tetraspanin-8	TSPAN8	2	2.50	NC	12.81	2.43E-04	Membrane	signal transducer activity	
(B) Cell-to-Cell Signaling										
P52907	F-actin-capping protein subunit alpha-1	CAPZA1	11	2.73	2.37	7.77	8.36E-07	Cytoplasm	N/A ^d	
P04233	CD74 antigen	CD74	10	NC	NC	NC	2.97E-09	Plasma Membrane	cytokine receptor activity	
P10909	Custerin	CLU	15	2.69	NC	2.47	4.08E-03	Membrane	protein binding	
P17844	Probable ATP-dependent RNA helicase DDX5	DDX5	8	ND	NC	NC	1.67E-03	Nucleus	hydrolase; transcription coactivator	
P98095	Fibulin-2	FBLN2	6	NC	NC	NC	8.96E-13	Extracellular	extracellular matrix binding	
P21333	Filamin-A	FLNA	283	4.78	29.2	9.21	2.66E-12	Cytoplasm	signal transducer activity	
P02751	Fibronectin	FN1	86	25.06	NC	NC	2.58E-05	Extracellular Matrix	extracellular matrix structural constituent	
P06396	Gelsolin	GSN	26	11.60	NC	NC	5.41E-07	Cytoplasm	calcium ion binding	
P12268	IMP dehydrogenase 2	IMPDH2	2	0.49	0.00	0.09	2.36E-05	Cytoplasm	IMP dehydrogenase activity	
P06756	Integrin alpha V/antigen CD51	ITGAV	6	25.52	NC	NC	2.99E-04	Plasma Membrane	calcium channel activity	
P13796	Plastin-2	LCP1	36	4.23	NC	9.29	8.83E-11	Plasma Membrane	regulation of intracellular protein transport	
P35749	Myosin-11	MYH11	116	NC	NC	NC	5.32E-10	Cytosol	ATP binding; structural constituent of muscle	
P43490	Nicotinamide phosphoribosyltransferase	NAMPT	8	NC	NC	NC	9.91E-04	Cytoplasm	Glycosyltransferase	
Q15149	Plectin	PLEC	478	9.26	30	13.48	9.38E-08	Cytoplasm	structural constituent of muscle	
Q9ULZ3	Apoptosis-associated speck- like protein containing a CARD	PYCARD	2	NC	NC	NC	7.24E-03	Mitochondrion/ER	cysteine-type endopeptidase activity	
P62906	60S ribosomal protein L10a	RPL10A	2	0.12	0.02	0.07	2.57E-07	Ribosome	RNA binding; structural constituent of ribosome	

Ratio of CD24 ⁺ PDAC to CD24 ⁻ ANT									
Accession	Protein name	Gene	Peptide ^a	Sample pair			P-value	Location	Function
				1	2	3			
Q9Y265	RuvB-like 1	RUVBL1	7	10.15	4.67	24.70	1.02E-04	Membrane	ATP binding; DNA helicase activity
Q9Y230	RuvB-like 2	RUVBL2	6	20.99	NC	NC	9.20E-04	Membrane	chromatin regulator; DNA helicase activity
P31949	S100 calcium binding protein A11	S100A11	15	NC	NC	33.61	4.91E-12	Cytoplasm	calcium ion binding
Q96Q15	Serine/threonine-protein kinase SMG1	SMG1	1	NC	NC	NC	1.13E-03	Nucleus/Cytoplasm	kinase activity; transferase
P04216	Thy-1 membrane glycoprotein/CD90 antigen	THY1	6	2.99	NC	NC	1.61E-04	Plasma Membrane	Rho GTPase activator activity
Q9UDY2	Tight junction protein ZO-2	TJP2	4	ND	NC	NC	1.87E-03	Plasma Membrane	guanylate kinase activity
P50552	Vasodilator-stimulated phosphoprotein	VASP	2	NC	NC	NC	2.16E-04	Plasma Membrane	T cell receptor
(C) Cellular Growth and Proliferation									
P35611	Alpha-adducin	ADD1	4	4.40	NC	NC	5.91E-03	Plasma Membrane	metal ion binding
Q99832	T-complex protein 1 subunit eta	CCT7	10	0.23	0.33	0.27	3.59E-04	Cytoplasm	ATP binding; chaperone
P32970	CD70 antigen	CD70	5	NC	NC	ND	3.87E-03	Membrane	signal transduction
P19835	Bile salt-activated lipase	CEL	4	0.03	0.00	0.00	7.03E-09	Secreted	Hydrolase
Q03692	Collagen alpha-1(X) chain	COL10A1	6	NC	NC	ND	1.52E-03	Extracellular	metal ion binding
Q05707	Collagen alpha-1(XIV) chain	COL14A1	143	5.68	NC	ND	6.92E-03	Extracellular	extracellular matrix structural constituent
Q86UP6	Transmembrane protein UO-44	CUZD1	0	0.00	0.00	0.00	1.10E-07	Membrane	trypsinogen activation
Q14118	Dystroglycan	DAG1	0	0.00	0.00	0.00	2.46E-03	Plasma Membrane	receptor; cell surface binding
O95571	Protein ETHE1, mitochondrial	ETHE1	6	ND	NC	NC	6.04E-03	Mitochondrion	hydrolase activity
Q9BWH2	FUN14 domain-containing protein 2	FUNDC2	0	0.00	0.00	0.00	3.17E-09	Mitochondrion	N/A
P19823	Inter-alpha-trypsin inhibitor heavy chain H2	ITIH2	2	NC	NC	ND	1.79E-03	Secreted	endopeptidase inhibitor activity
Q6PKG0	La-related protein 1	LARP1	0	0.00	0.00	0.00	6.18E-04	Nucleus	RNA binding
P07237	Protein disulfide-isomerase	P4HB	73	0.29	0.27	0.39	1.05E-12	Plasma Membrane	protein disulfide isomerase activity
Q9Y617	Phosphoserine aminotransferase	PSAT1	0	0.00	0.00	0.00	8.18E-09	Cytosol	Aminotransferase
Q71UM5	40S ribosomal protein S27-like	RPS27L	0	0.21	0.21	0.00	2.52E-08	Nucleus	Ribonucleoprotein; translation activator activity
P31947	14-3-3 protein sigma/Stratifin	SFN	34	NC	NC	NC	8.79E-08	Cytoplasm/Nucleus	protein kinase C inhibitor activity
Q01082	Spectrin beta chain	SPTBN1	86	2.20	8.54	2.11	3.14E-04	Plasma Membrane	phospholipid binding; structural constituent of cytoskeleton

Accession	Protein name	Gene	Peptide ^a	Ratio of CD24 ⁺ PDAC to CD24 ⁻ ANT			P-value	Location	Function
				Sample pair					
				1	2	3			
P08240	Signal recognition particle receptor subunit alpha	SRPR	0	0.00	0.00	0.04	2.50E-08	Membrane	receptor; signal recognition particle binding

Note:

^aThe number of peptide spectra for the target protein was from a single LC-MS/MS run of CD24⁺ PDAC sample.

^bNC, not calculable (number/0). NC and 0.00 represent that the protein was identified only in PDAC or ANT samples, respectively.

^cND, not detected.

^dN/A, not available.