Published in final edited form as:

J Proteome Res. 2012 April 6; 11(4): 2272–2281. doi:10.1021/pr201059g.

Identification of Glycoprotein Markers for Pancreatic Cancer CD24⁺CD44⁺ Stem-like Cells Using nano LC-MS/MS and Tissue Microarray

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Abstract

Pancreatic adenocarcinoma is characterized by late diagnosis due to lack of early symptoms, extensive metastasis, and high resistance to chemo/radiation therapy. Recently, a subpopulation of cells within pancreatic cancers, termed cancer stem cells (CSCs), has been characterized and postulated to be the drivers for pancreatic cancer and responsible for metastatic spread. Further studies on pancreatic CSCs are therefore of particular importance to identify novel diagnosis markers and therapeutic targets for this dismal disease. Herein, the malignant phenotype of pancreatic cancer stem-like CD24⁺CD44⁺ cells was isolated from a human pancreatic carcinoma cell line (PANC-1), and demonstrated 4-fold increased invasion ability compared to CD24[−]CD44⁺ cells. Using lectin microarray and nano LC-MS/MS, we identified a differentially expressed set of glycoproteins between these two subpopulations. Lectin microarray analysis revealed that fucose- and galactose-specific lectins, UEA-1 and DBA respectively, exhibit distinctly strong binding to CD24⁺CD44⁺ cells. The glycoproteins extracted by multi-lectin affinity chromatography were consequently analyzed by LC-MS/MS. 17 differentially expressed glycoproteins were identified, including upregulated Cytokeratin 8/CK8, Integrin β 1/CD29, ICAM1/CD54 and Ribophorin 2/RPN2, and downregulated Aminoamidase N/CD13. Immunohistochemical analysis of tissue microarrays showed that CD24 was significantly associated with late-stage pancreatic adenocarcinomas, and RPN2 was exclusively coexpressed with CD24 in a small population of CD24-positive cells. However, CD13 expression was dramatically decreased along with tumor progression, preferentially present on the apical membrane of ductal cells and vessels in early-stage tumors. Our findings suggest that these glycoproteins may provide potential therapeutic targets and promising prognostic markers for pancreatic cancer.

Keywords

glycoprotein; pancreatic cancer; stem-like cells; CD24; LC-MS/MS; lectin affinity chromatography; tissue microarray

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Supporting Information Available: Carbohydrate specificities of the 15 lectins used for lectin microarray (Table S1). Detailed clinical characteristics of normal pancreas and pancreatic adenocarcinoma samples in tissue microarrays (Table S2 and S3). Glycoproteins identified from the CD24⁺CD44⁺ and CD24[−]CD44⁺ PANC-1 cells with FDR < 0.01 after UEA-1, DBA and AAL lectins enrichment (Table S4 and Table S5). Summary of immunohistochemical analysis of CD24, RPN2 and CD13 expression in 13 normal pancreas tissues and 53 pancreatic adenocarcinomas (Table S6). A representative base peak chromatogram of lectin-captured fraction from CD24⁺CD44⁺ cells and MS/MS spectrum of a peptide from Ribophorin-2 (Figure S1). Subcellular location of glycoproteins identified from CD24⁺CD44⁺ cells and CD24[−]CD44⁺ cells (Figure S2). Western blotting analysis of CD13 in whole cell lysates from CD24⁺CD44⁺ cells and CD24[−]CD44⁺ cells with or without enzymatic deglycosylation using PNGase F (Figure S3).

Introduction

Pancreatic adenocarcinoma is currently the fourth deadliest cancer in the United States, with an overall 5-year survival rate of 1–4% and a median survival period of 4–6 months.^{1–2} It is typically diagnosed at a late stage, frequently after metastasis, and is notoriously resistant to chemo/radiation therapy.³ Discovery of new early detection markers and efficient therapeutic targets still remains a major challenge.

Increasing evidence has verified that solid tumors, including pancreatic cancer, are initiated and sustained by a subset of cancer stem cells (CSCs).^{4–10} CSCs have characteristic traits such as a distinct surface marker expression profile and the intrinsic capacity to self-renew and recapitulate the phenotype of the original tumor.¹¹ Putative pancreatic CSCs, defined first by the simultaneous expression of CD44, CD24, and epithelial-specific antigen (ESA), are highly tumorigenic and possess the properties of self-renewal and the ability to produce differentiated progeny.⁹ Further studies on CSCs offer the possibility to find early detection markers and to develop novel therapeutic targets that can improve therapeutic efficacy for pancreatic cancer.

Glycosylation is one of the most common posttranslational modifications in proteins. The alterations in protein glycosylation which occur through varying the heterogeneity of glycosylation sites or changing glycan structure of proteins on the cell surface have been shown to correlate with the development of cancer and other disease states.^{12–13} Most clinical biomarkers and CSCs surface markers in solid tumors are glycoproteins,^{11, 14} suggesting that altered glycosylation may be important in CSC identification and/or function. Lectin microarrays^{15–16} coupled with lectin affinity chromatography¹⁷ have greatly facilitated the profiling of glycan expression patterns of glycoproteins.^{18–19} The enriched glycoproteins or glycopeptides can be consequently determined using mass spectrometry, which plays an essential role in the analysis of glycoproteins.^{20–21}

Herein, we present a glycoproteomics comparison of two subpopulations isolated from PANC-1 cells, one of the most aggressive human pancreatic carcinoma cell lines, using the stemness markers CD24 and CD44. We demonstrated that PANC-1 cells were nearly all positive for CD44; therefore, the comparison was investigated between CD24⁺CD44⁺ and CD24[−]CD44⁺ cells. We utilized matrigel invasion assay to explore the invasion ability of the two subpopulations, and lectin microarrays to detect differences in their glycosylation patterns. The glycoproteins extracted by multi-lectin affinity chromatography were identified by LC-MS/MS and quantified using a label-free method termed normalized spectral index. Seventeen differentially expressed glycoproteins were identified in CD24⁺CD44⁺ cells compared to CD24[−]CD44⁺ cells. The differential expression was further validated by western blot and evaluated by immunohistochemistry on tissue microarrays including 13 normal human pancreas tissues and 53 pancreatic adenocarcinomas to investigate the correlation with clinicopathologic parameters. Our findings may provide promising prognostic markers and potential therapeutic targets for pancreatic cancer.

Materials and Methods

Cell Culture

PANC-1 cells were cultured in DMEM/F12 (Gibco, Invitrogen) supplemented with 10% (v/v) FBS (fetal bovine serum; Invitrogen), 100 units/mL penicillin and 0.1 mg/mL streptomycin. Adherent cells were maintained in standard conditions for less than 8 passages at 37°C with 5% CO₂ and detached using trypsin/EDTA solution (trypsin 0.25% and 1mM EDTA).

FACS

PANC-1 cells were grown to 80–90% confluence prior to flow sorting. The cells were trypsinized for dissociation, washed twice with FACS buffer (PBS containing 1% BSA and 1mM EDTA), and resuspended in FACS buffer at a concentration of 10^6 cells/100 μ L. Direct-labeled antibodies, anti-CD24-FITC and anti-CD44-PE (BD Bioscience, Pharmingen), were added to the sample at a dilution of 1:40 and incubated with the sample in the dark for 20 min on ice. Subsequently, cells were washed twice with FACS buffer and resuspended in FACS buffer followed by flow cytometry using a FACS Aria (BD Immunocytometry Systems, Franklin Lakes, NJ). Side scatter and forward scatter profiles were used to eliminate cell doublets. Cells were reanalyzed for purity, which typically was >96%.

Matrigel Invasion Assay

In vitro invasion assays were performed using 24-well Matrigel-coated transwell chambers with 8 μ m pore size (BD Biosciences, Bedford, MA). CD24⁺CD44⁺ and CD24⁺CD44⁺ cells were isolated by flow sorting in PBS containing 2% FBS. After sorting, cells were washed twice with PBS and seeded in serum-free media at a density of 5×10^4 cells per well on the upper matrigel chambers. Media containing 10% FBS was placed in the lower chamber as a chemoattractant. After 24 hr of incubation at 37 °C in a 5% CO₂ atmosphere, cells on the upper surface of the filter were removed. The invading cells on the underside were examined under a Nikon inverted microscope at $\times 200$ magnification and counted by choosing five high power fields randomly. All experiments were performed in triplicate. Statistical significance was calculated using a student's t-test.

Protein Extraction

Cells from both populations were washed twice with PBS and then suspended in ice-cold lysis buffer containing 1% octyl-D-glucopyranoside, 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 $\times$ protease inhibitor cocktail. The cells were homogenized with 30 strokes in a Dounce glass homogenizer with a tight-fitting pestle, and then incubated on ice for 10 min with periodic vortex. The cell lysate was centrifuged at 40,000g for 30 min at 4 °C. The supernatant was collected and the protein concentration was determined by the Bradford method.²²

Lectin Microarray

The Lectin microarrays were produced as described previously.¹⁷ The SNA-2 and TKA lectins were purchased from E.Y. Laboratories (San Mateo, CA) and the other thirteen lectins were purchased from Vector Laboratories (Burlingame, CA). Their carbohydrate specificities are listed in Supplementary Table S1. Lectins were dissolved in PBS at a concentration of 1 mg/mL and spotted on FAST[®] 16 pad nitrocellulose slides (Whatman Inc., Florham Park, NJ) using a piezoelectric noncontact printer (Nano plotter; GESIM). Each lectin was printed in triplicate and each spot contained 2.0 nL lectin, with spotting of 400 pL for five times. The slides were incubated in a humidity-controlled incubator (> 45% humidity) overnight to allow lectin immobilization.

The glycoprotein-lectin interaction was explored using a biotin-streptavidin system as described before.¹⁵ Four micrograms of protein from cell lysate were labeled with EZ-link iodoacetyl- LC-biotin (Pierce) for 90 min, followed by adding 1 μ L 2-mercaptoethanol (Sigma) to quench the reaction. The slides were blocked with 1% BSA/PBS for 1 hr, then the labeled sample was added to lectin blocks and incubated for 1 hr. After washing with PBST, the slides were incubated with streptavidinylated fluorescent dye Alexa555 (Invitrogen Biotechnology) for 1 hr, followed by three washes with PBST, and then dried by

centrifugation prior to imaging. The fluorescence intensity of each spot was explored under a microarray scanner (Genepix 4000A; Axon) and analyzed by Genepix® Pro 6.0 software.

Lectin Affinity Chromatography

The Pierce centrifuge column (Thermo Scientific, IL) was packed with agarose-bound lectins, containing Ulex Europaeus Agglutinin I (UEA-1), Dolichos Biflorus Agglutinin (DBA), and Aleuria Aurantia Lectin (AAL), 0.5 mL slurry each. All lectins were purchased from Vector Laboratories (Burlingame, CA). The column was washed with 3 mL of binding buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1mM CaCl₂). Cell lysates from both populations, containing one milligram of proteins respectively, were diluted four times with ice-cold binding buffer, loaded onto the column and incubated for 15 min. The column was washed with 6 mL of binding buffer to remove non-specific binding proteins. The captured glycoproteins were then released twice with 2 mL of elution buffer (100 mM L-fucose and 200 mM N-acetylgalactosamine in binding buffer, pH 7.4). The eluted fractions were concentrated using Microcon YM-3 centrifugal filter devices to 200 μ L in 50 mM NH₄HCO₃.

Tryptic Digestion and PNGase F Treatment

The glycoproteins were reduced with 5 mM TCEP (Thermo) for 30 min at 37 °C, alkylated with 15 mM iodoacetamide (Sigma) in the dark at room temperature for 30 min, and then digested with trypsin (Promega, Madison, WI) at 37 °C overnight. The glycopeptides were deglycosylated by PNGase F (New England Biolabs, Ipswich, MA) at 37 °C for 16 hr, and then dried using a SpeedVac concentrator (Thermo Savant, Milford, MA).

LC-MS/MS

The peptide mixtures were resolubilized in 0.1% formic acid and analyzed by an LTQ mass spectrometer (Thermo Finnigan, San Jose, CA). Chromatographic separation of peptides was explored on a Paradigm MG4 micropump system (Michrom Biosciences Inc., Auburn, CA) with a flow rate of 300 nL/min. Peptides were separated by a C18 separation column (0.1 mm $\times$ 150 mm, C18 AQ particles, 5 μ m, 120 Å, Michrom Biosciences Inc., Auburn, CA). Both solvents, A (water) and B (acetonitrile), contained 0.1% formic acid. A 90 min linear gradient was applied from 5% to 40% B. The MS instrument was operated in positive ion mode. The spray voltage was set at 1.5 kV, the capillary voltage was 30 V and the capillary temperature was 150 °C. The normalized collision energy was set at 35% for MS/MS. For each cycle of one full mass scan (range of m/z 400–2000), the five most intense ions in the spectrum were selected for tandem MS analysis, unless they appeared in the dynamic or mass exclusion lists. The data were acquired in data-dependent mode using the Xcalibur software (2.1.0 build 1139).

Data Analysis

All MS/MS spectra were searched using SEQUEST algorithm 29 incorporated in Proteome Discoverer software version 1.1.0.263 (Thermo Finnigan) against the UniProt human protein database Release 2010_11. The search was performed using the following parameters: (1) Fixed modification, Carbamidomethyl of C; (2) variable modification, oxidation of M and Asn→Asp; (3) allowing two missed cleavages; (4) peptide ion mass tolerance 1.50 Da; (5) fragment ion mass tolerance 1.0 Da; (6) peptide charges +1, +2, and +3. False discovery rate (FDR) was set to be < 0.05 or < 0.01 for each database search. The Xcorr values for all the charge states (+1, +2, and +3) were automatically adjusted to achieve the predetermined FDR value. If multiple proteins share the same peptide sequences, they will be reported as a protein group. The peptides were identified with a probability cutoff of $p > 0.99$, and proteins with a probability higher than 0.90.

Proteins identified from CD24⁺CD44⁺ and CD24⁻CD44⁺ PANC-1 cells were quantified using a label-free quantitative method termed the normalized spectral index (SI_N), which combines spectral count and fragment ion intensity.²³ SI_N is defined as the sum of fragment ion intensities for all spectra counted for a protein (*SI*) normalized by the sum of *SI* over all proteins and by the length of the protein.²³ SI_N fold-change of a given protein was calculated as the ratio of the average SI_N for the protein between CD24⁺CD44⁺ and CD24⁻CD44⁺ cells. A 2-fold change was considered as a significant difference between the two samples.

Western Blot Analysis

Fifteen micrograms of proteins from CD24⁺CD44⁺ and CD24⁻CD44⁺ cell lysates were separated by 4–15% SDS-PAGE and then transferred to PVDF membranes (Bio-Rad, CA). After transfer, the membranes were blocked for 2 hr by 1% BSA in PBST, and then incubated with the following primary antibodies: mouse anti-CD24, mouse anti-CD13 and rabbit anti-RPN2 from Abcam (Cambridge, MA), rabbit anti-CD44 from Millipore (Temecula, CA) and rabbit β-Actin from Cell Signaling (Boston, MA). After being washed with PBST three times, the membranes were incubated with peroxidase-conjugated anti-IgG (H+L) for 1 hr, washed with PBST three times, and then detected by Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific, IL).

Tissue Samples

The tissue microarrays (TMAs) of formalin-fixed paraffin-embedded pancreatic tumors and normal tissue were purchased from US Biomax Inc. (Catalog No. PA484 and PA1002). Tissue specimens included 13 normal human pancreas tissues (age: from 21 to 50 years, median: 35 years) and 53 cases of pancreatic ductal adenocarcinoma tissues (age: from 34 to 78 years, median: 57 years), identified with clinical stages and pathology grades. The clinical characteristics are listed in Supplementary Table S2 and S3. The images of the TMAs stained with hematoxylin-eosin (H&E) were provided on the website of US Biomax Inc.. The tissue samples originated from different donors and each sample had 2 replicates.

Fluorescent Immunohistochemistry

The paraffin-embedded 5 μm tissue microarrays were dewaxed in xylene for 10 min twice and rehydrated through a series of alcohol solutions (100% ethanol twice, 90% ethanol, 70% ethanol, 5 min each) 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 staining between the primary antibodies and the tissues.

To achieve immunofluorescence staining, mouse anti-CD13 (Abcam, Cambridge, MA) or a mixture of mouse anti-CD24 (R&D Systems Inc., Minneapolis, MN) and rabbit anti-RPN 2 (Abcam, Cambridge, MA) was incubated with the TMAs overnight at 4 °C at a dilution of 1:100. Then DyLight 488 anti-rabbit IgG (H+L) and DyLight 549 anti-mouse IgG (H+L) (Vector laboratories, Burlingame, CA) were diluted (1:200) and incubated with the TMAs for 1 hr at room temperature. The nuclei visualization was explored by DAPI counterstaining. Between each step, there were three washes in PBST for 5 min each. Finally, the TMAs were dehydrated in alcohol (70% ethanol, 90% ethanol, 100% ethanol twice, 5 min each), and coverslipped using a CC/MountTM permanent mounting medium (Sigma).

Results and Discussion

A diagram of the workflow is shown in Figure 1. First, the pancreatic cancer stem-like cells were enriched from PANC-1 cells by flow cytometry according to stemness markers CD24 and CD44. Since nearly 100% PANC-1 cells expressed CD44 (see FACS results below), the comparison was investigated between CD24⁺CD44⁺ and CD24⁻CD44⁺ cells. Matrigel assay was applied to investigate their invasion abilities and lectin microarray was employed to detect their aberrant glycosylation patterns. Based on the results obtained by lectin microarrays, three lectins, UEA-1, DBA, and AAL, were selected to capture the glycoproteins using affinity chromatography. The glycoproteins were subsequently identified by nano LC-MS/MS and quantified by the spectral index. Finally, the differentially expressed glycoproteins were validated by western blot and immunohistochemistry of tissue microarrays including 13 normal pancreas tissues and 53 cases of pancreatic adenocarcinomas.

FACS

The pancreatic cancer stem-like cells were isolated from PANC-1 cells based on stemness markers. CD24, CD44 and epithelial-specific antigen (ESA) have been demonstrated as surface markers for pancreatic CSCs.⁹ PANC-1, a pancreatic ductal epithelial cell line, maintains ductal epithelial features²⁴, the cancer stem-like cells were therefore enriched by sorting for CD24⁺CD44⁺ subpopulation. Flow cytometry was employed to investigate the expression of CD24 and CD44 in PANC-1 cells. As shown in Figure 2, 12.15% of PANC-1 cells expressed both CD24 and CD44. Note that CD24⁺ cells were all positive for CD44. In addition, nearly 100% PANC-1 cells expressed CD44, which was consistent with the results reported in other pancreatic cancer cell lines, such as MiaPaCa, BxPC-3 and L3.6pl cells.¹⁰ Since PANC-1 cells are nearly all CD44 positive, the analysis of CD24⁺CD44⁺ cancer stem-like cells was investigated in comparison with CD24⁻CD44⁺ cells. The two subpopulations were isolated and enriched by FACS for the subsequent experiments.

Matrigel Invasion Assay

CD24 is a cell surface protein which is overexpressed in a variety of solid tumors and is involved in cell adhesion and tumor metastasis.^{25–26} CD24 overexpression has been reported to support the rolling of tumor cells on endothelial monolayers²⁷ and promote tumor cell motility and invasion *in vivo*.²⁸ In order to investigate the involvement of CD24 in PANC-1 cell invasion, CD24⁺CD44⁺ and CD24⁻CD44⁺ PANC-1 cells were seeded respectively in serum-free media on the upper Matrigel-coated transwell chambers with 8-μm pore size membrane. After 24-hr incubation, the number of invading CD24⁺CD44⁺ cells increased significantly, exhibiting about 4-fold increase in comparison to CD24⁻CD44⁺ cells ($p < 0.0001$) (Figure 3). This observation demonstrated that the expression of CD24 distinctly increases the invasion capacity of PANC-1 cells.

Profiling of glycan signatures by Lectin Microarray

Fifteen lectins, with a broad range of carbohydrate recognition, were applied to profile different glycan signatures between CD24⁺CD44⁺ and CD24⁻CD44⁺ cells by using lectin microarrays. The carbohydrate specificities of the 15 lectins are shown in Table S1. As shown in Figure 4, UEA-1, DBA and AAL displayed much higher binding abilities to both subset cells than the other 12 lectins. In addition, UEA-1 and DBA lectins exhibited much stronger binding capacity to CD24⁺CD44⁺ cells compared to CD24⁻CD44⁺ cells, with significant differences in fluorescent intensity ($p < 0.001$). UEA-1 and AAL have similar carbohydrate specificities to fucose. UEA-1 preferentially binds to (α-1,2) linked fucose residues, while AAL specifically recognizes (α-1,3)/(α-1,4) and (α-1,6) fucose residues.^{29–30} DBA specifically binds to α-linked N-acetylgalactosamine (GalNAc). This result implied

much higher expression of fucosylated and galactosylated glycoproteins in CD24⁺CD44⁺ cells. Generally, alternations in the fucosylation and sialylation levels are the most common tumor-associated glycan aberrations.^{31–32} Herein, our findings indicated that fucosylation and galactosylation changes may differentially occur in pancreatic CSCs, and UEA-1 and DBA may distinguish the glycoproteins between CD24⁺CD44⁺ and CD24[−]CD44⁺ cells.

Glycoprotein Identification

The lectin affinity chromatography was performed to capture glycoproteins from CD24⁺CD44⁺ and CD24[−]CD44⁺ cells by using a multi-lectin column. Lectin microarray results showed that UEA-1, DBA and AAL displayed much higher binding abilities than the other 12 lectins and the binding partners of UEA-1 and DBA may contain potential markers to distinguish CD24⁺CD44⁺ from CD24[−]CD44⁺ cells. Therefore, agarose-bound UEA-1, DBA and AAL were packed into a column to capture the glycoproteins of the two subsets. The eluted glycoproteins from three independent enrichment experiments were digested by using trypsin and the glycopeptides were released by PNGase F, followed by LC-MS/MS analysis using an LTQ mass spectrometer. As an example, a representative base peak chromatogram of a lectin-captured fraction from CD24⁺CD44⁺ cells and a typical MS/MS spectrum are shown in Supplementary Figure S1.

All MS/MS spectra were searched using SEQUEST algorithm 29. A total of 66 glycoproteins were identified from CD24⁺CD44⁺ cells (Supplementary Table S4) and 59 glycoproteins identified from CD24[−]CD44⁺ cells (Supplementary Table S5), when the FDR was set to < 0.01. The detailed information for each glycoprotein, including spectral count, unique peptide counts and the sum of fragment ion intensities, is listed in Supplementary Table S4 and S5. The number of glycoproteins identified in this work was relatively low compared to previous studies using Con A or WGA to capture glycoproteins from cells,³³ mainly because of much narrower specificity of UEA-1, DBA, and AAL than that of Con A and WGA. CD24 was not observed in the mass spectra, probably due to the low solubility and low tryptic digestion efficiency of this small protein, which is composed of 27–35 amino acids. However, CD24 expression was exclusively confirmed in the CD24⁺CD44⁺ cells by western blotting.

The subcellular locations of the glycoproteins were annotated based on the UniProt database. A majority of these glycoproteins were localized to the membrane, endoplasmic reticulum, and lysosome. However, the subcellular distribution patterns were different between these two subset cells (Supplementary Figure S2). Both endoplasmic reticulum and lysosomal glycoproteins increased in CD24⁺CD44⁺ cells compared to CD24[−]CD44⁺ cells. The endoplasmic reticulum glycoproteins increased from 13 (22%) to 18 (27%), and lysosomal glycoproteins increased from 15 (25%) to 20 (30%), as determined by MS spectral data.

Differentially Expressed Glycoproteins Analyzed by the Label-Free Spectral Index Method

The glycoproteins identified from CD24⁺CD44⁺ and CD24[−]CD44⁺ cells were quantified using a label-free normalized spectral index method, which combines three MS abundance features: peptide count, spectral count and fragment ion intensity. This approach can largely eliminate variances between replicate MS measurements, permitting quantitative reproducibility and can be used to estimate the abundance of proteins in a complex cellular lysate.²³ It has proved to be more statistically robust than other label-free methods such as spectral count (SC), area under the curve (AUC) and peak area measurements.^{23, 34}

A total of 17 differentially expressed glycoproteins between CD24⁺CD44⁺ and CD24[−]CD44⁺ cells were identified. The detailed information of these proteins is listed in

Table 1. Although the cut off value of significant difference between the two subpopulations was 2-fold change in spectral index (SI_N), we also took into consideration proteins of interest with a ratio of 1.5 or greater. Among these differentially expressed proteins, are 6 membrane proteins and 9 lysosomal proteins. Note that 13 differentially expressed glycoproteins were upregulated in CD24⁺CD44⁺ cells, including Cytokeratin 8/CK8, Integrin β 1/CD29, ICAM-1/CD54 and Ribophorin 2/RPN2. The other 4 glycoproteins were down-regulated: CD13 antigen, Cathepsin Z, Galectin-3-binding protein and Beta-galactosidase. The ratio of the spectra index of CD44 protein between the CD24⁺CD44⁺ and CD24⁻CD44⁺ cells was 1.176 (Supplementary Table S4 and S5), which was consistent with flow cytometry results, suggesting nearly identical CD44 level in the two subpopulations.

Accumulated evidence suggests that CK8, CD29, and CD54 are associated with increased tumor invasiveness and disease progression in a variety of cancers. CK8 belongs to a group of proteins known as intermediate filaments that make up the cytoskeletal structure of epithelial cells and play a major role in maintaining cell morphology and structural integrity.³⁵ CK8 has been reported to correlate with malignancy in carcinomas of the head and neck³⁶ and as an indication of poor prognosis in squamous cell carcinomas of the oral cavity and the esophagus.^{37–38} Integrin β 1/CD29 is a member of the integrin family, which are heterodimeric transmembrane receptors that mediate cellular interactions with the extra cellular matrix (ECM). *In vitro* and *in vivo* experiments showed that β 1 integrin expression is critical for the initiation of mammary tumorigenesis and maintenance of tumor growth.³⁹ Moreover, the expression of β 1 integrin is strongly associated with cell adhesion and metastasis in pancreatic cancers⁴⁰. CD54, also known as intercellular adhesion molecule-1 (ICAM-1), is a cell surface glycoprotein of the immunoglobulin superfamily and widely expressed in tumor, stromal, and immune cells.⁴¹ It is up-regulated in many types of cancers including pancreatic cancer⁴², and may activate intracellular signaling pathways in cancer cells leading to enhanced cell motility, invasion and metastasis.^{43–44} Consistent with these observations, the upregulation of CK8, CD29 and CD54 in CD24⁺CD44⁺ cells with the increased invasion ability indicated that they may promote the invasion of pancreatic cancer cells and might be considered as potential therapeutic targets for pancreatic cancer, especially in preventing the metastasis of this highly aggressive disease.

Ribophorin 2/RPN2 is a type I integral membrane protein in the rough endoplasmic reticulum (RER), which is part of an N-oligosaccharyl transferase complex and plays an important role either in translocation or in the maintenance of RER.⁴⁵ It has also been reported to play a key role in drug-resistant tumor cells and act as a facilitator, stabilizing factor for N-glycosylation of P-glycoprotein.⁴⁶ The downregulation of the gene RPN2 can efficiently induce apoptosis of docetaxel-resistant human breast cancer cells.⁴⁶ Herein, RPN2 was upregulated in CD24⁺CD44⁺ cells, indicating that it may be correlated with the drug resistance of pancreatic cancer stem cells. Aminopeptidase N (APN/CD13) is a transmembrane ectopeptidase expressed on a wide variety of cells. It is a multifunctional protein and plays varying roles in cell migration, cell proliferation, cell differentiation, cytokine and neuropeptide regulation, and may contribute to the regulation of angiogenesis.^{47–49} In this study, CD13 expression was significantly downregulated in CD24⁺CD44⁺ cells, which might be involved in the functional regulation of pancreatic cancer stem cells.

Western Blotting Analysis of Selected Proteins

To further verify the changes of the glycoproteins listed in Table 1, four proteins, CD24, CD44, CD13 and Ribophorin-2 (RPN2), were selected for validation by using Western blotting (Figure 5). It should be noted that CD24 was exclusively observed in CD24⁺CD44⁺ cells, proving the high purity of CD24⁺CD44⁺ cells isolated from FACS. In addition, the expression of CD44 showed no significant difference between CD24⁺CD44⁺ and

CD24⁺CD44⁺ cells, which was in good agreement with the results of flow cytometry and MS spectra. The up-regulation of RPN2 and down-regulation of CD13 in CD24⁺CD44⁺ cells were also confirmed, which were consistent with the label-free quantitation results shown in Table 1. In addition, western blotting analysis of CD13 after enzymatic deglycosylation with PNGase F (Supplementary Figure S3) revealed that CD13 is heavily glycosylated in both subpopulations. The intensity of the deglycosylated CD13 in CD24⁺CD44⁺ cells remained significantly lower than that in CD24⁺CD44⁺ cells, which further demonstrated that the difference of CD13 in the two subpopulations was from the change in protein abundance. To elucidate their roles in pancreatic adenocarcinoma, the expression of CD24, RPN2 and CD13 was further evaluated by immunohistochemical analysis.

Immunohistochemistry analysis of Selected Proteins in both Normal Pancreas and Pancreatic Adenocarcinoma

Tissue microarrays, containing 13 normal human pancreas tissues and 53 pancreatic adenocarcinomas, were employed to evaluate the expression of CD24, RPN2 and CD13 and investigate their association with histological grade and other clinicopathologic data. The distribution of protein expression was evaluated as the percentage of stained cells, which was > 80%, 40–80%, 10–40%, < 10%, or no staining. The specimens with ≥ 10% positive cells were classified as positive and as negative when it was < 10%, according to the previous study.⁵⁰ The immunohistochemical analysis of CD24, RPN2 and CD13 expression in normal and pathological pancreatic tissues were summarized in Supplementary Table S6.

The double immunohistochemical staining was performed to identify the expression of CD24 and RPN2 in normal pancreas and pancreatic adenocarcinomas. Both CD24 and RPN2 showed sparse expressions in acinar cells of normal pancreas tissues, with less than 5% CD24⁺ cells and 2% RPN2⁺ cells respectively (Figure 6A), which were considered negative as described above. Note that the ductal epithelia in normal pancreas tissues exhibited no expression of CD24 and RPN2 (Figure 6A). In the pancreatic adenocarcinomas, the positive rates of CD24 and RPN2 were 75.5% (40/53) and 24.5% (13/53), respectively. CD24 was observed in the cytoplasm and membrane of tumor cells as reported recently⁵¹, showing a significantly higher expression (Figure 6E and 6F) and higher rate of positivity (Supplementary Table S6) in late-stage and high-grade tumors. The predominant expression of CD24 in late-stage tumors further confirmed the high invasion ability of CD24⁺ tumor cells, as observed in the matrigel assay. Jacob et al.⁵² reported that CD24 was expressed in 71.6% pancreatic adenocarcinomas and strongly correlated with higher tumor grades. These findings suggest that CD24 may be a promising marker to evaluate the progression of pancreatic adenocarcinoma. As shown in Figure 6B–6F, RPN2 showed a heterogeneous distribution in pancreatic adenocarcinomas. It should be noted that RPN2 was exclusively coexpressed with CD24 in a small population of CD24 positive cells, with a non-overlapping pattern at the subcellular level. Since RPN2 siRNA has been reported to significantly reduce drug-resistant tumor growth,⁴⁶ the exclusive coexpression of RPN2 and CD24 in pancreatic cancer suggests that this population could be of particular interest as a therapeutic target to treat pancreatic adenocarcinoma.

The immunohistochemistry analysis of CD13 showed that 100% (13 of 13) of normal pancreas samples were positive for CD13, which was abundantly expressed on the apical membrane surface in the centroacinar regions (Figure 7A, 7B). The percentage of CD13⁺ cells within the normal tissue specimen was greater than 80%. However, CD13 expression was significantly decreased along with the progression of tumor stages (Supplementary Table S6). 37.7% (20 of 53) pancreatic adenocarcinomas were positive for CD13, with the percentage of positive cells less than 20%. Note that CD13 was preferentially expressed on the apical membrane of ductal cells and vessels in early-stage tumors (Figure 7C and 7D),

which is consistent with the important role of CD13 in angiogenesis.^{53–54} CD13 participates in angiogenesis generating and modulating angiogenic signals and is essential for capillary tube formation, as a marker of angiogenic vessels.^{53–55} The positivity rate of CD13 in late-stage pancreatic adenocarcinoma was dramatically reduced (Supplementary Table S6), showing weak or no staining for CD13 (Figure 7E and 7F). This result suggests that the decrease of CD13 expression significantly correlates with tumor progression, and the dominant expression of CD13 in cancerous ducts and vessels may provide a promising prognostic marker for early-stage pancreatic adenocarcinoma.

Conclusion

In this work, we identified a differentially expressed set of glycoproteins between pancreatic cancer CD24⁺CD44⁺ stem-like cells and CD24[−]CD44⁺ cells using lectin microarray and LC-MS/MS. The two subpopulations were enriched from a human pancreatic carcinoma cell line (PANC-1). Almost all PANC-1 cells expressed CD44, however, only 12.15% cells were double positive for CD24 and CD44. CD24⁺CD44⁺ cells exhibited 4-fold increased invasion ability relative to CD24[−]CD44⁺ cells, suggesting that CD24 may promote pancreatic cancer cell invasion. Lectin microarray revealed significantly increased fucosylated and galactosylated glycoproteins in CD24⁺CD44⁺ cells. Alteration of fucosylation level is one of the most common tumor-associated glycan aberrations.³¹ Our findings indicated that both fucosylation and galactosylation changes occur in pancreatic CSCs. 17 differentially expressed glycoproteins were identified in CD24⁺CD44⁺ cells, including up-regulated Cytokeratin 8/CK8, integrin β 1/CD29, ICAM1/CD54, and RPN2 and down-regulated APN/CD13.

We further evaluated the expressions of CD24, RPN2, and CD13 by immunohistochemical analysis of tissue microarrays and investigated their correlation with clinicopathologic parameters in pancreatic adenocarcinoma. The predominant expression of CD24 in late-stage and high-grade tumors further confirmed the increased invasion ability of CD24-positive cells, suggesting that CD24 may be a promising marker to evaluate the progression of pancreatic adenocarcinoma. The exclusive coexpression of RPN2 and CD24 in pancreatic adenocarcinoma suggested that this population could be of particular interest for a therapeutic target to treat this dismal disease. CD13 was abundantly expressed on the apical membrane surface of centroacinar cells in normal pancreas; however, the expression was significantly decreased along with the progression of tumor stages. Consistent with the important role of CD13 in angiogenesis generation^{53–54}, CD13 was preferentially expressed on the apical membrane of cancerous ducts and vessels in early stages, which may provide CD13 as a promising prognostic marker for pancreatic adenocarcinoma. Our findings may not only have implications for studies of CSCs, but also provide potential therapeutic targets and prognostic markers of tumor progression for pancreatic cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded under the National Institutes of Health Grants R01 49500 (D.M.L.) and R21 CA134623 (D.M.L.). We thank Dr. Lidong Wang (University of Michigan) for providing the PANC-1 cell line and Dr. Xiaolei Xie (University of Michigan) for technical assistance with the mass spectrometry.

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Figure 1. Workflow for the identification of glycoproteins differentially expressed between CD24⁺CD44⁺ and CD24⁻CD44⁺ PANC-1 cells.

Figure 2.

Flow cytometry analysis of CD24 and CD44 expression in PANC-1 cells. (A) A dot plot of CD44-PE against CD24-FITC fluorescence. (B, C) Histograms for CD24-FITC and CD44-PE fluorescence, respectively. Only 12.15% PANC-1 cells are double positive for CD24 and CD44. Note that CD24⁺ PANC-1 cells are also positive for CD44, and PANC-1 cells are almost all positive for CD44.

Figure 3.
In vitro invasion assay. (A) Photomicrographs showing CD24⁺CD44⁺ and CD24⁻CD44⁺ PANC-1 cells that migrated through the Matrigel-coated filter after 24 hr incubation. Scale bar, 50 μm. (B) Graphical representation of the mean and s.d. of invading CD24⁺CD44⁺ and CD24⁻CD44⁺ cells in five independent fields. The number of CD24⁺CD44⁺ invading cells was significantly higher than that of CD24⁻CD44⁺ cells ($p < 0.0001$).

Figure 4.
Lectin Microarray. A) A typical lectin microarray image showing the binding patterns of CD24⁺CD44⁺ and CD24⁻CD44⁺ cells to 15 lectins. Each lectin was spotted in triplicate. B) A Graph representing the mean and s.d. of fluorescent intensity of the 15 lectins. **p* < 0.001.

Figure 5.

Western blotting analysis of CD24, CD44, CD13, Ribophorin-2/RPN2 and Actin. Five micrograms of proteins from CD24⁺CD44⁺ and CD24⁻CD44⁺ cells was separated by 4–15% SDS-PAGE and transferred to PVDF membranes. Note that CD24 was exclusively expressed in CD24⁺CD44⁺ cells, while CD44 showed no significant difference between the two subpopulations.

Figure 6.

Immunofluorescence double staining with CD24 (red) and RPN2 (green) in human normal pancreas (A) and pancreatic adenocarcinoma of early (B–D) and late stages (E, F). (A) Both CD24 and RPN2 showed sparse expression in the acinar regions, but no expression in the ductal epithelia (*). (B) A weak staining for both CD24 and RPN2 in stage I pancreatic cancer. (C, D) More frequent expression of CD24 and slightly increased RPN2 expression in stage II pancreatic cancer. The insert in C is a higher magnification showing the coexpression of RPN2 and CD24. (E, F) Tissues of late-stage pancreatic adenocarcinoma showing prominent CD24 expression, but no (E) or weak staining (F) for RPN2. CD24 was strongly expressed in the cytoplasm and membrane of cancer cells. Note that RPN2 was exclusively coexpressed with CD24 in a small population of CD24 positive cells, but no overlap in the subcellular components. A–F= ×200 magnification.

Figure 7.

Distribution of CD13 (red) in human normal pancreas (A, B) and pancreatic adenocarcinoma of early (C, D) and late stages (E, F). (A, B) CD13 was abundantly expressed on the apical membrane surface in centroacinar regions in normal pancreas. (C, D) CD13 expression was dramatically decreased in early-stage tumors, preferentially localized in cancerous ducts (C) and vessels (D). The insert in C is a higher magnification showing CD13 expressed on the apical membrane of ductal epithelium. (E, F) No or weak staining for CD13 in late-stage tumors. CD13-negative malignant ducts (E). Weak and sparse CD13 staining in stage IV cancer (F). A–F = $\times 200$ magnification.

Table 1

Differentially Expressed Glycoproteins between CD24⁺CD44⁺ and CD24⁺CD44⁺ cells.

Accession	Protein name	Gene	Location	Ratio ^a	p-value ^b
CATD_HUMAN	Cathepsin D	CTSD	Lysosome	1.51	8.54E-07
K2C8_HUMAN	Cytokeratin 8	KRT8	Cytoplasm	6.66	5.62E-04
FUCO_HUMAN	Alpha-L-fucosidase I	FUCA1	Lysosome	NA ^c	6.40E-04
CATH_HUMAN	Pro-cathepsin H	CTSH	Lysosome	NA	8.13E-04
AMPN_HUMAN	CD13 antigen	ANPEP	Membrane	0.32	0.0010
PPGB_HUMAN	Lysosomal protective protein	CTSA	Lysosome	3.10	0.0012
CATZ_HUMAN	Cathepsin Z	CTSZ	Lysosome	0 ^d	0.0035
LG3BP_HUMAN	Galectin-3-binding protein	LGALS3BP	Extracellular	0.25	0.0047
BGAL_HUMAN	Beta-galactosidase	GLB1	Lysosome	0.53	0.0067
MPRL_HUMAN	CD222 antigen	IGF2R	Lysosome & Membrane	1.64	0.0069
ITB1_HUMAN	Integrin β1/CD29 antigen	ITGB1	Membrane	3.41	0.0089
RPN2_HUMAN	Ribophorin-2	RPN2	ER ^e & Membrane	1.67	0.0089
NPC2_HUMAN	Epididymal secretory protein E1	NPC2	Secreted	3.09	0.0098
LAMP1_HUMAN	CD 107a antigen	LAMP1	Membrane	1.98	0.017
ICAM1_HUMAN	CD54 antigen	ICAM1	Membrane	2.83	0.025
DIAC_HUMAN	Di-N-acetylchitinobiase	CTBS	Lysosome	2.68	0.026
GALNS_HUMAN	N-acetylgalactosamine-6-sulfatase	GALNS	Lysosome	2.73	0.030

^aRatio of the spectral index of a target protein identified from CD24⁺CD44⁺ cells compared to that from CD24⁺CD44⁺ cells.

^bp-value: statistical significance of differentially expressed proteins between CD24⁺CD44⁺ and CD24⁺CD44⁺ cells. The values were generated by Student's *t*-test for three independent experiments.

^cNA means the protein was identified only in CD24⁺CD44⁺ cells.

^d0 means the protein was not identified in CD24⁺CD44⁺ cells.

^eER represents endoplasmic reticulum.