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Discovery of putative pancreatic cancer biomarkers using subcellular proteomics

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ABSTRACT

Pancreatic cancer (PC) is a highly aggressive disease that frequently remains undetected until it has progressed to an advanced, systemic stage. Successful treatment of PC is hindered by the lack of early detection. The application of proteomic analysis to PC combined with subcellular fractionation has introduced new possibilities in the field of biomarker discovery. We utilized matched pairs of pancreas tumor and non-tumor pancreas from patients undergoing tumor resection. The tissues were treated to obtain cellular protein fractions corresponding to cytosol, membrane, nucleus and cytoskeleton. The fractions were then separated by molecular weight and digested with trypsin, followed by liquid chromatography and tandem mass spectrometry. The spectra obtained were searched using Sequest engine and combined into a single analysis file to obtain a semi-quantitative number, spectral count, using Scaffold software. We identified 2393 unique proteins in non-tumor and cancer pancreas. Utilizing PLGEM statistical analysis we determined 104 proteins were significantly changed in cancer. From these, we further validated four secreted proteins that are up-regulated in cancer and have potential for development as minimally-invasive diagnostic markers. We conclude that subcellular fractionation followed by gel electrophoresis and tandem mass spectrometry is a powerful strategy for identification of differentially expressed proteins in pancreatic cancer.

1. Introduction

Pancreatic Cancer (PC) is a highly aggressive malignant disease with a median survival rate of less than 6 months. The major reason for poor outcome is the progression of the disease at time of diagnosis [1]. Due to the fact that PC is typically asymptomatic until the disease has progressed to a virtually incurable stage, research is needed to enhance early detection strategies. Current strategies utilize the molecular marker CA19-9. However, this marker is not specific to pancreatic cancer, is not expressed at high levels early in disease progression, and is not universally expressed in PC patients [2,3]. The need exists to identify a more specific and highly sensitive marker, or set of markers, for the early detection of this deadly disease.

The successful treatment of PC has historically also been limited, with the only successful treatment option being surgical resection of the tumor in its early stages [2]. Recurrence in patients is high and local and distant spread of the disease is usually already present at the time of initial diagnosis. Furthermore, some PC patients are not candidates

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for surgical removal of the tumor due to proximity of the tumor to the superior mesenteric artery [4].

Post-surgical and adjuvant therapies have not been much improved over the past decade. Combinatorial approaches have been studied in which chemotherapy plus radiation has been shown to modestly improve the overall survival rate. More sophisticated studies utilizing molecular targeting strategies have not proven much more successful in enhancing survival [4,5]. New strategies are needed to treat the systemic and recurrent characteristics of pancreatic cancer pathology.

The development of mass spectrometric techniques for identification and quantification of proteins has introduced new possibilities in the field of biomarker discovery [6–11]. The identification of proteins directly associated with tumor microenvironment can provide insight into the molecular pathogenesis and progression of the disease. These molecules might also represent potential therapeutic targets. Identification of PC-specific proteins in biological fluids such as blood and urine can lead to minimally invasive screening tests, enabling early detection of disease. The development of biomarker capabilities can also lead ultimately to molecular methods of disease staging, prognosis and development of patient-specific treatment strategies.

One of the major challenges in proteomics studies for biomarker discovery is the large dynamic range of proteins found in tissue samples. Strategies to decrease the complexity of such samples are required in order to generate meaningful identification and semi-quantification of the proteins within tissue. One particular approach has been the use of subcellular fractionation [12–14]. Separating the proteins from a tissue sample into fractions based on their location within the cell serves to decrease the complexity of the sample, allowing the researcher to delve into the lower-abundant proteome for identification of disease related proteins. Our research has employed the use of subcellular fractionation with mass spectrometric techniques to identify potential biomarkers of pancreatic cancer. Our approach has been to use these strategies as a screening mechanism (Fig. 1), comparing the subcellular fractionation and proteomic profile from non-tumor and diseased tissue. Using spectral count combined with statistical modeling, we have determined a subset of the identified proteins to be up-regulated in cancer. Of these, we have chosen four for further validation. Immunohistochemistry has verified the expression of three of these proteins, while western blotting has confirmed the increased expression of all four of these proteins in cancer. Based on these results we believe subcellular fractionation, in conjunction with mass spectrometry based proteome profiling, provides a useful screening strategy in the search for disease biomarkers.

2. Methods and materials

2.1. *Sample collection*

Five patients presenting to Carolinas Medical Center for treatment of pancreatic disease were recruited and consented to participate in this study based on Institutional Review Board (IRB) approved protocols. When participants underwent surgery for resection, the pancreas was sent to the pathology department for staging. At this time the pathologist dissected a part of the pancreas tumor as well as a part of non-tumor adjacent pancreas tissue from each patient and snap froze it in liquid nitrogen. All samples collected were inventoried in the Liver-Biliary-Pancreatic tissue repository located at Cannon Research Center. The repository made these tissues available for study through an IRB approved research protocol. Each patient's medical record was reviewed and the clinical pathology for each patient was recorded (Table 1).

2.2. *Subcellular fractionation*

Preliminary studies assessing the effectiveness of the Proteoextract SPEK kit (Calbiochem) were carried out on HEPG2

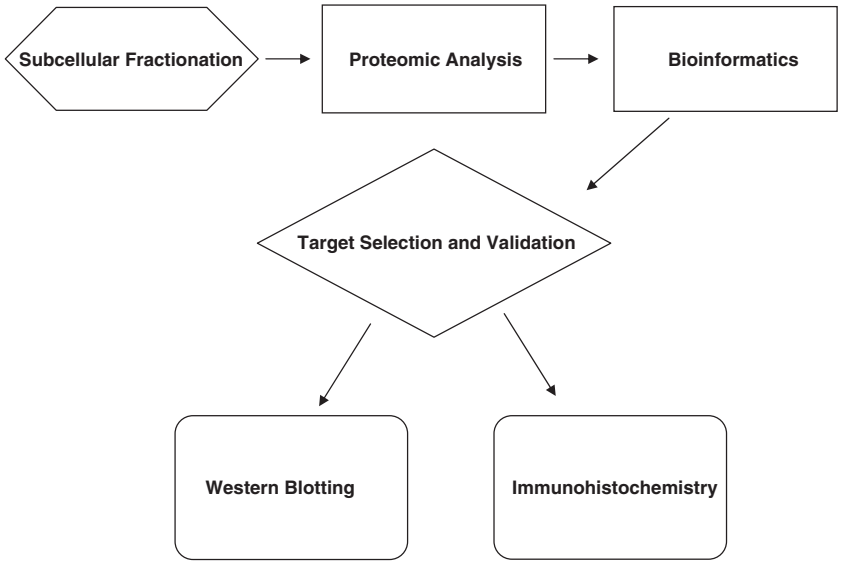

Fig. 1 – Schematic diagram showing the work flow for subcellular fractionation and proteome analysis for biomarker identification.

Table 1 – Pathology of patient participants.

ID	Diagnosis	Grade	Stage	Metastases	Gender	Age
30	Invasive adenocarcinoma	Moderately differentiated G2	pT3 N1 Mx	Metastatic to lymph nodes	Male	69
44[a]	Invasive adenocarcinoma	Moderately differentiated G2-G3	pT3 N1 Mx	Metastatic to lymph nodes	Male	60
47	invasive adenocarcinoma	Well to moderately differentiated G1-2	pT1 N0 MX	no evidence of LN metastasis	Female	63
69[a]	Invasive adenocarcinoma	Moderately to poorly differentiated G2-3	pT3 N1 MX	Metastatic to lymph nodes	Male	70
101	Adenocarcinoma	Moderately to poorly differentiated intermed. to high	T4N1 Mx	Metastatic to lymph nodes	Male	58

[a] Samples have been used for proteomic analysis.

cultured cells according the published SPEK protocol. Fractionation products were subjected to western blot analysis using fraction specific antibodies and demonstrated little to no carryover between fractions (Supplemental Fig. S1). Patient samples were then processed using the SPEK kit as recommended by the manufacturer.

While on dry ice, approximately 50 mg tissue was excised from snap frozen non-tumor pancreas tissue and pancreas tumor from the five patients (here referred to by study ID numbers as 30N, 30T, 44N, 44T, 47N, 47T, 101N, 101T and 69N 69T). The Proteoextract SPEK kit was utilized for subcellular fractionation based on the solubility of the respective subcellular compartments. Each tissue sample was minced using a scalpel and the pieces were transferred to a separate 2.0 ml tube then washed with 1× ice-cold phosphate buffered saline (PBS) on ice 2 times for 2 minutes each time. PBS was removed and 1.0 ml extraction buffer I (EB-I) + 5.0 μl PIC (Calbiochem) was added. Samples were homogenized using a TH115 homogenizer (Omni International) for 30 s to 1 min until tissue was completely dissociated. The samples were then incubated on ice 10 min. The homogenate was centrifuged at 1000×*g* for 20 min at 4 °C. Supernatant was transferred to a clean tube and labeled Fraction 1 (cytosol enriched fraction). To the pellet was added 1.0 ml EB-II plus 5 μl protease inhibitor cocktail (PIC). Pellets were resuspended and incubated while shaking at 4 °C for 30 min then centrifuged at 5000×*g* at 4 °C for 10 min. Supernatant was transferred to a clean tube and labeled fraction 2 (membrane enriched fraction). To the pellet was added 0.5 ml EB-III plus 5 μl PIC and 1.5 μl Benzonase. The pellet was resuspended and incubated while shaking at 4 °C 10 min. The samples were then centrifuged at 7000×*g* for 10 min at 4 °C. Supernatant was transferred to a clean tube and labeled fraction 3 (nuclear enriched fraction). To the pellet was added 0.5 ml EB-IV and 5 μl PIC. The pellet was resuspended and this sample labeled fraction 4 (cytoskeleton enriched fraction). Protein quantitation was carried out using the microplate procedure from the BCA assay (Thermo) using a 1:10 dilution of each fraction.

2.3. *Total protein extraction*

While on dry ice, 50 mg of tissue was excised from snap frozen non-tumor pancreas and pancreas tumor from each of the five patients. Tissue was minced using a scalpel and washed with 1× PBS twice, on ice, for 2 min each time. 1.0 ml 1× radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1.0% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris–Cl pH 8.0) with 2× protease inhibitor cocktail (Roche Applied Science) was added and samples homogenized for 30 s to one minute until tissue was completely dissociated. Samples were then incubated on ice for 30 min. After incubation samples were centrifuged at 4 °C and 12,000×*g* for 15 min. Supernatant was transferred to a clean tube and spin repeated. The final supernatant was transferred again to a clean tube. Protein quantification was carried out on a 1:10 dilution of each sample using the microplate procedure and the BCA assay kit (Thermo).

2.4. *One-dimensional SDS-PAGE and In-gel digestion*

The subcellular fractionation products from two matched pairs (44N/T and 69N/T) were chosen for LC-MS/MS analysis based on their optimal protein concentration post fractionation. These patients were both male, of similar age and identical stage/grade of disease. Both patients had demonstrated metastasis to lymph nodes. From the eight subcellular fractions of each of these two patients, 30 micrograms of protein was loaded onto a 4–12% Bis–Tris gel and electrophoresed at 35 mA for 1.5–2 h. Gels were washed with de-ionized water, then fixed in 50% methanol, 7% acetic acid for 15 min. Post-fixing, gels were rinsed with de-ionized water then stained using Gelcode Blue stain (Thermo) for 30 min to one hour. Destaining was accomplished with deionized water. Gels were imaged on a Fuji LAS3000 (Supplemental Fig. S2).

Gel lanes were dissected into 20 slices. Gel slices were chopped to 1 mm^3 pieces and destained using three 20 min incubations/changes of 50% acetonitrile, 25 mM ammonium bicarbonate. Once all stain was removed, gel pieces were dehydrated in 100% acetonitrile and then dried in a Centrivap (Labconco) for 10 min or to complete dryness.

Gel pieces were re-hydrated in 50 mM ammonium bicarbonate containing 12.5 ng/μl Trypsin (Promega) on ice for 30 min, then incubated at 37 °C overnight. For extraction of peptides from the gel pieces, 100 μl 50% acetonitrile, 5% formic acid was added and samples were incubated while shaking at room temperature for 20 min. Samples were centrifuged at 14,000×*g* for 5 min and supernatant transferred to a clean tube. These steps were repeated for a total of 3 extractions of peptides from the gel pieces. The extraction product was dried to completion in the Centrivap (Labconco) and re-suspended in mass spectrometry analysis buffer (5% acetonitrile, 3% formic acid).

2.5. *LC-MS/MS, database search, and statistical analysis*

The resultant 80 samples per matched pair were analyzed in duplicate on a Thermo LTQ-XL Orbitrap mass spectrometer equipped with a Waters NanoACQUITY UPLC. Samples were separated by a 65 min linear gradient from 90% Solvent I (0.1% formic acid in water)/Solvent II (0.1% formic acid in acetonitrile) to 50% Solvent I/II at a flow rate of 500 nl/min on reversed phase chromatography using a trap/elute method with a C_{18} sample trap in line with a C_{18} analytical column [15]. Mass spectrometric analysis included analysis of the top 8 most abundant ions and 120 s dynamic exclusion. Spectra were searched against the human IPI version 3.18 database using the SEQUEST search algorithm from the Bioworks software (Thermo) with the following parameters; parent mass tolerance of 10 ppm, fragment tolerance of 0.5 Da, variable modification on methionine of 16 Da, and maximum missed cleavage of 2 [16]. Search results were entered into Scaffold software (Proteome Software) for compilation, normalization, and comparison of spectral counts, the number of identified peptides [17–19]. Protein identifications were made at the peptide probability of 95% and protein probability of 99%. Single peptide hits were excluded from analysis. The spectral count data from duplicate analyses of non-tumor and tumor samples were then compared using a power law global error model (PLGEM) in order to identify statistically significant protein changes between non-tumor and tumor samples [20,21]. PLGEM software was downloaded from www.bioconductor.org and run with the default false positive rate of .001, using raw spectral count as input. While PLGEM was developed using a normalized spectral abundance factor (NSAF) as input[14], its performance with a limited number of replicates has been shown to improve when raw spectral count rather than NSAF is used [22]. False discovery rates for PLGEM-generated significance lists were estimated using the Benjamini–Hochberg estimator [23]. The proteins identified as significantly changed in cancer were then compared to a database of 3020 blood plasma proteins that were previously identified in human plasma with two or more peptides [24,25]. Additional comparisons were made across biological samples (44 compared to 69) as well as across pathological status (non-tumor compared to tumor) for each subcellular fraction.

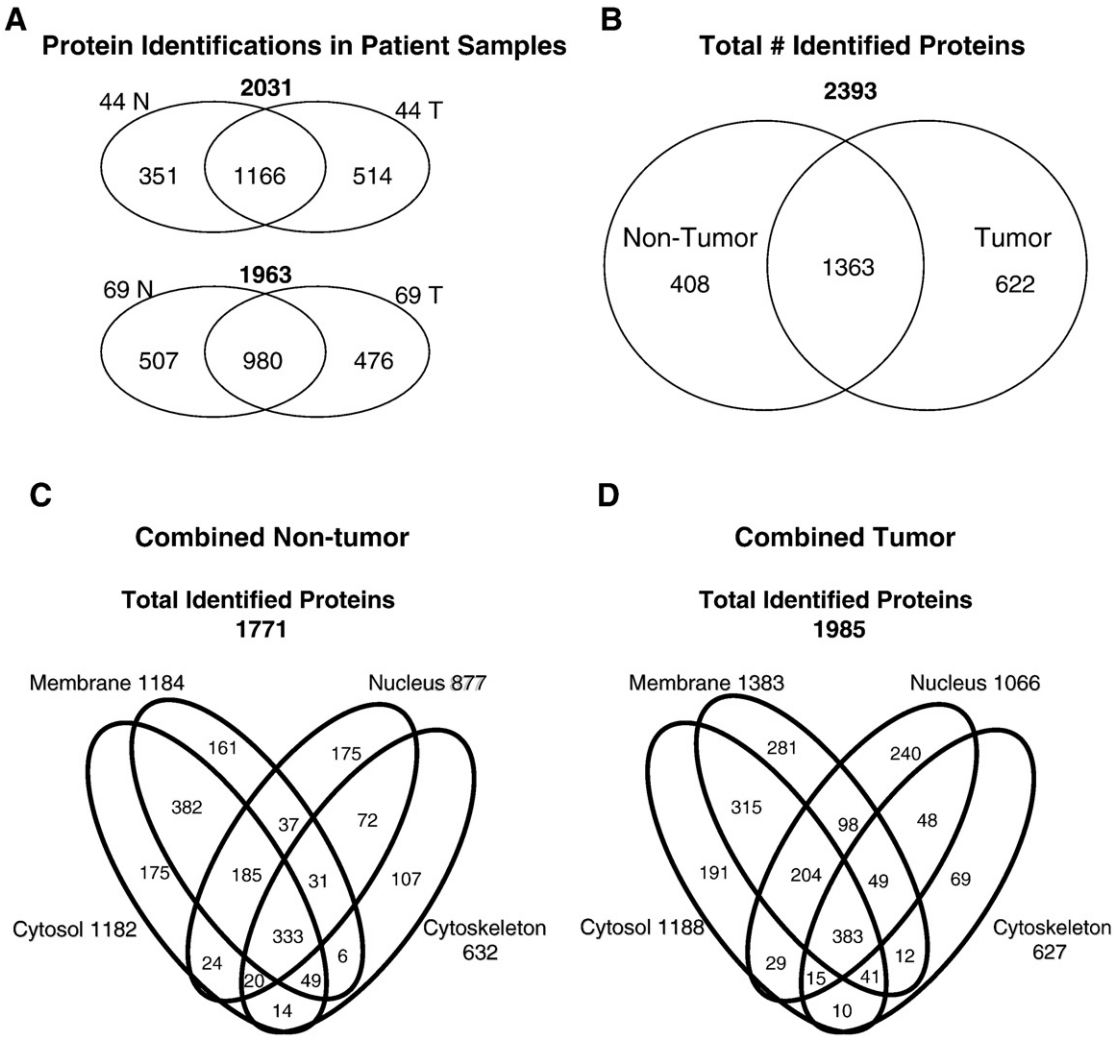

Fig. 2 – Venn Diagrams representing A: the number of proteins identified from each patient; B: the combined number of proteins identified in Non-tumor versus Tumor; and C–D: the proteins identified in each subcellular fraction.

2.6. Western blot analysis

15 μg of RIPA lysate or subcellular fractionation product was loaded onto a 4–12% Tris–Glycine NuPAGE gel (Invitrogen) and electrophoresed at 150 V for 80 min. Proteins were transferred to nitrocellulose membrane at 10–12 V overnight at 4 °C or at room temperature and 25 V for 2 h using the Invitrogen Xcell II blot module. Transfer efficiency (and in the case of fractionation products, evenness of sample loading) was confirmed by ponceau S staining of the membrane. Membranes were blocked for one hour at RT to overnight at 4 °C using 5% ECL blocking reagent (GE Healthcare) in tris-buffered saline containing 0.1% Tween 20 (TBST). Membranes were incubated in primary antibody at 1:500 dilution in blocking buffer for 2.5 h at room temperature to overnight at 4 °C. Blots were washed 3 times for 5 min and then incubated in the appropriate secondary antibody conjugated to HRP for one hour at room temperature. Blots were then washed again at 3 times 5 min. Chemiluminescent detection was accomplished using the ECL Advance kit from GE and a Fuji LAS3000 digital imager. GAPDH

Table 2 – Human plasma proteins with significant changes in pancreatic cancer tissue.

ID	Description	Non-Tumor		Cancer		STN[a]	Frac. #[b]	
		1	2	1	2			
IPI00292530	Inter-alpha-trypsin inhibitor heavy chain H1 precursor	0	2	82	107	4.985	1	
IPI00217966	Lactate dehydrogenase A	20	21	38	225	4.034	1	[c]
IPI00026314	Isoform 1 of Gelsolin precursor	20	25	111	160	3.991	1	[c]
IPI00220644	Isoform M1 of Pyruvate kinase isozymes M1/M2	11	29	26	230	3.991	1	
IPI00010951	Epiplakin	0	0	3	93	3.645	1	
IPI00013508	Alpha-actinin-1	53	66	112	300	3.605	1	[c]
IPI00021439	Actin, cytoplasmic 1	69	65	166	274	3.574	1	[c]
IPI00018769	Thrombospondin-2 precursor	0	0	13	78	3.551	1	[c]
IPI00164623	Complement C3 precursor	37	29	165	112	3.460	1	[c]
IPI00298994	Talin-1	15	15	84	95	3.414	1	[c]
IPI00305461	Inter-alpha-trypsin inhibitor heavy chain H2 precursor	3	7	39	66	3.290	1	[c]
IPI00021428	Actin, alpha skeletal muscle	0	0	30	47	3.266	1	[c]
IPI00296099	Thrombospondin-1 precursor	0	0	4	72	3.245	1	[c]
IPI00009904	Protein disulfide-isomerase A4 precursor	149	238	30	48	−4.224	1	
IPI00027230	Endoplasmin precursor	288	429	84	134	−4.284	1	[c]
IPI00015133	Chymotrypsinogen B precursor	117	145	16	0	−4.913	1	[c]
IPI00021447	Alpha-amylase 2B precursor	143	524	0	0	−8.433	1	
IPI00021048	Isoform 1 of Myoferlin	3	0	72	157	3.603	2	[c]
IPI00020557	Low-density lipoprotein receptor-related protein 1 precursor	2	0	44	69	2.888	2	[c]
IPI00021812	Neuroblast differentiation-associated protein AHNAK (Fragment)	0	0	52	45	2.734	2	[c]
IPI00024284	Basement membrane-specific heparan sulfate proteoglycan precur.	0	2	47	35	2.567	2	[c]
IPI00032258	Complement C4-A precursor	11	6	111	22	2.425	2	[c]
IPI00296337	Isoform 1 of DNA-dependent protein kinase catalytic subunit	89	47	9	3	−2.665	2	
IPI00019502	Myosin-9	2	6	60	147	3.341	3	[c]
IPI00006114	Pigment epithelium-derived factor precursor	6	9	87	110	2.997	3	
IPI00022200	alpha 3 type VI collagen isoform 1 precursor	51	56	311	162	2.789	3	[c]
IPI00009342	Ras GTPase-activating-like protein IQGAP1	12	15	62	145	2.689	3	
IPI00018219	Transforming growth factor-beta-induced protein ig-h3 precursor	0	2	16	59	2.393	3	[c]
IPI00022229	Apolipoprotein B-100 precursor	0	0	31	41	2.351	3	[c]
IPI00299738	Procollagen C-endopeptidase enhancer 1 precursor	0	0	29	37	2.264	3	[c]
IPI00298497	Fibrinogen beta chain precursor	6	0	58	21	2.167	3	[c]
IPI00021885	Isoform 1 of Fibrinogen alpha chain precursor	0	0	45	12	2.119	3	[c]
IPI00030363	Acetyl-CoA acetyltransferase, mitochondrial precursor	111	141	29	13	−2.633	3	[c]
IPI00022418	Isoform 1 of Fibronectin precursor	139	45	288	1145	6.721	4	[c]
IPI00032328	Isoform HMW of Kininogen-1 precursor	0	0	149	0	3.943	4	[c]
IPI00013079	EMILIN-1 precursor	0	0	6	109	3.495	4	
IPI00298281	Laminin gamma-1 chain precursor	156	192	111	0	−2.706	4	
IPI00022200	Alpha 3 type VI collagen isoform 1 precursor	1600	2122	1194	1203	−2.766	4	[c]
IPI00218725	laminin alpha 2 subunit precursor	252	236	177	0	−2.782	4	
IPI00026944	Isoform 1 of Nidogen-1 precursor	93	201	81	0	−2.790	4	
IPI00329327	Isoform 2 of Extracellular matrix protein FRAS1 precursor	10	67	0	0	−2.896	4	
IPI00013976	Laminin beta-1 chain precursor	147	230	104	0	−3.051	4	[c]
IPI00022822	Isoform Long of Collagen alpha-1(XVIII) chain precursor	138	115	13	0	−4.308	4	
IPI00024284	Basement membrane-specific heparan sulfate proteoglycan core protein pre	669	715	260	119	−4.884	4	[c]
IPI00013933	Isoform DPI of Desmoplakin	215	186	6	2	−5.669	4	

[a] Signal-to-noise (STN) of PLGEM (power law global error model): false positive rate cutoff of 0.001.
[b] Fraction number: Cytosol (1), Membrane (2), Nucleus (3), and Cytoskeleton (4).
[c] Also correlates to Pancreatic Cancer Biomarker Compendium 20.

(Santa Cruz) was used as a loading control. Additional primary antibodies used are from Santa Cruz (Thrombospondin-2, PEDF, IGH3) and from Abcam (Biglycan).

2.7. *Immunohistochemistry*

Paraffin embedded pancreas cancer tissue blocks for each patient were obtained from the pathology department at Carolinas Medical Center and four micron sections were cut onto slides. Tissue microarray slides were obtained from US Biomax (catalog # PA481 and BC00113). Slides were deparaffinized in xylene and hydrated through graded alcohols to dH_2O. The remainder of the procedure was performed using the AutostainerPlus (Dako). Endogenous peroxidase was blocked using 3% H_2O_2 (Sigma). Slides were incubated for one hour with primary antibody at the following concentrations: Biglycan (Abcam) 2 μg/ml; PEDF (Genway) 5 μg/ml; Thrombospondin-2 (Santa Cruz) 4 μg/ml. IgG from the species in which the antibody was produced was used as a negative control. Secondary antibody was followed by peroxidase-conjugated streptavidin (Dako) for 10 min and DAB (Dako) for 5 min. Slides were removed from stainer, rinsed in water, counterstained with light green, dehydrated, cleared and mounted with resinous mounting media. Slides were imaged using an Olympus BX51 microscope equipped with an Olympus DP70 camera and DP controller imaging software.

3. Results

3.1. *Subcellular fractionation and proteome analysis*

The subcellular fractionation combined with mass spectrometry analysis workflow generated 2031 total protein identifications in patient 44 (351 proteins specific to non-tumor tissue and 514 unique to cancer); and 1963 protein identifications in patient 69 (507 non-tumor and 476 tumor specific) (Fig. 2A). Combining this data into a non-redundant data set, we identified a total of 2393 proteins in non-tumor and cancer pancreas tissue, with 408 of these proteins unique to non-tumor pancreas tissues, 622 unique to the pancreas cancer samples and 1363 proteins common to both tissue types

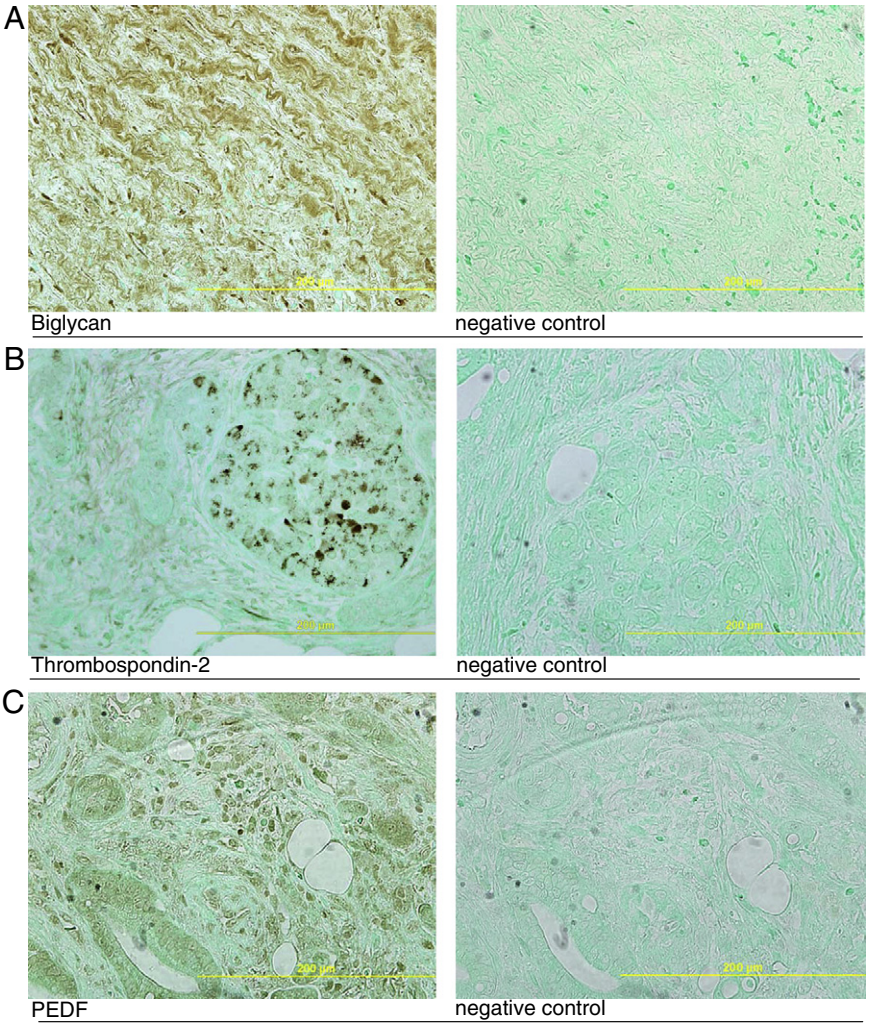

Fig. 3 – Representative photographs of Immunohistochemical staining of formalin fixed paraffin embedded patient tumor tissue with respective antibodies and no antibody in negative controls. (all are 400× magnification) A: Biglycan, B: Thrombospondin-2, C: PEDF.

(Fig. 2B, Supplemental Table s1). Each subcellular fraction showed unique protein identifications which attest to the efficacy of the Proteoextract SPEK kit for the snap frozen pancreas tissue (Fig. 2C–D). Additionally, comparison of the combined data generated across biological replicates for non-tumor tissue shows 70% overlap while tumor tissue demonstrates 58% overlap (Supplemental Fig. S3A). This data also supports the reproducibility of the technique. When the data are combined such that all identifications in one patient are compared to the other patient, the overlap is 86% which illustrates the homogeneity in the patient population (Supplemental Fig. S3B).

PLGEM analysis with raw spectral count rather than normalized spectral abundance factor identified numerous proteins exhibiting significant differential expression in non-tumor versus tumor tissues (Supplemental Table S2). The false discovery rate based on a false positive rate of .001 on PLGEM analysis was 1.4%. Of the 104 significantly changed proteins in our samples, 43 proteins were identified within the published 3020 human plasma proteome (Table 2) [24,25]. These 43 proteins were then cross referenced with a recent compilation of reported proteins and genes throughout the scientific literature thought to play a role in the pathogenesis of PC [26]. The comparison demonstrated 65% overlap, indicating a good correlation between our data and PC, while at the same time showing that this data has uncovered novel targets for further study.

3.2. *Target identification and validation*

Statistically significant increases in proteins associated with the pathogenesis of cancer were of particular interest in our study. Using PLGEM statistical modeling we identified cancer associated up-regulation in the proteins Biglycan (BGN), Pigment Epithelium-derived Factor (PEDF) Thrombospondin-2 (THBS-2) and TGF-β induced protein ig-h3 precursor (βIGH3). All have been identified in connection with cancer progression, as players in tumor microenvironment, cell proliferation or angiogenic processes. Immunostaining of associated patient tumor tissue sections verified the positive expression of three of these proteins of interest in situ (Fig. 3).

Western blotting of RIPA total protein lysates from four patient-matched tumor tissue pairs confirmed increased expression of the target proteins in cancer when compared to non-tumor tissue (Fig. 4A). When subcellular fractions were submitted to western blot analysis utilizing the PEDF antibody, the results demonstrate expression is most significant in the nuclear fraction of the tumor tissues (Fig. 4B).

3.3. *Functional translocation of PEDF*

PEDF, an anti-angiogenic factor, has been associated with inhibition of the proliferation of pancreatic cancer cells [27]. This seems at odds with our results. However we have observed that in non-tumor pancreas tissue PEDF is expressed in the cytoplasm, while in cancerous lesions the PEDF is more prominent in the nucleus (Fig. 5B, 6). PEDF expression on a multiple tissue array including malignant and non-tumor tissue exhibited notable staining in brain and prostate cancer, however this staining is not localized to the nucleus as seen in pancreas cancer (Fig. 5).

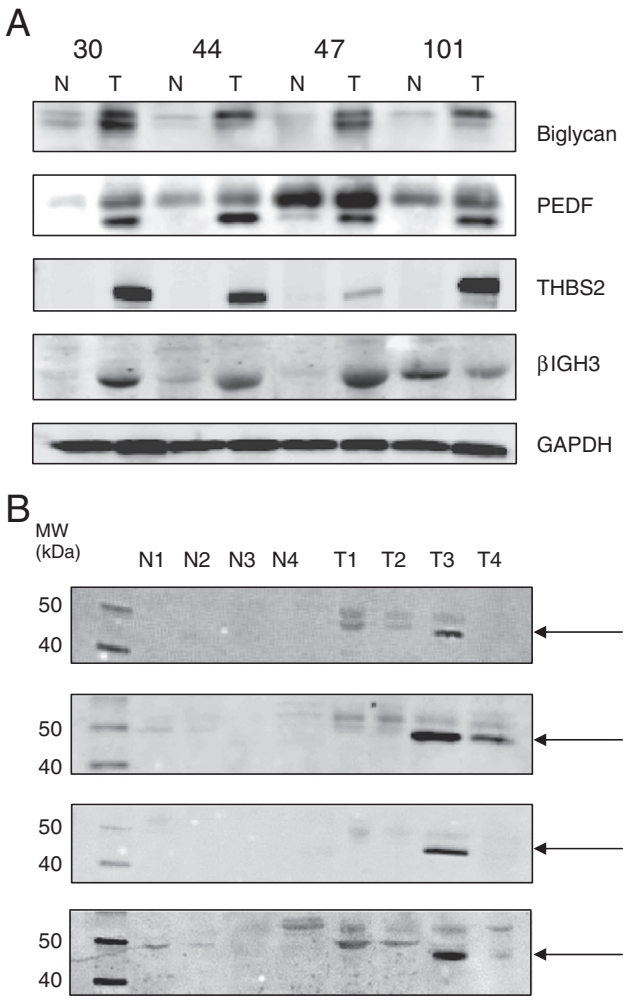

Fig. 4 – A) Western blot analysis RIPA total protein lysates demonstrating increased expression of Biglycan, PEDF, Thrombospondin-2 and bIGH3 in Tumor (T) tissues vs. non-tumor (N) from study patients. GAPDH is used as a loading control. B) Western Blot analysis of PEDF expression in subcellular fractions of non-tumor and tumor samples from study patients shown from top to bottom as patient # 30, 44, 47, 69. PEDF (indicated by arrows) is most abundant in the nuclear fraction of tumor tissues. Fraction numbers 1, 2, 3, 4 represent each cyotosol, membrane, nucleus, and cytoskeleton, respectively.

4. Discussion

The dire nature of pancreatic cancer has generated a great deal of proteomics research in recent years. Most studies have utilized 2-dimensional gel electrophoresis, differential gel electrophoresis and/or SELDI-TOF strategies for identification of protein changes within the pancreatic cancer proteome. These strategies by nature do not provide a global proteomic profile of this disease. The number of protein changes identified in 2-D gel/ LC-MS studies is typically quite low (fewer than 50 proteins identified), while studies targeted at plasma or pancreatic juices are not reflective of tumor microenvironment and thus potentially eliminate possible

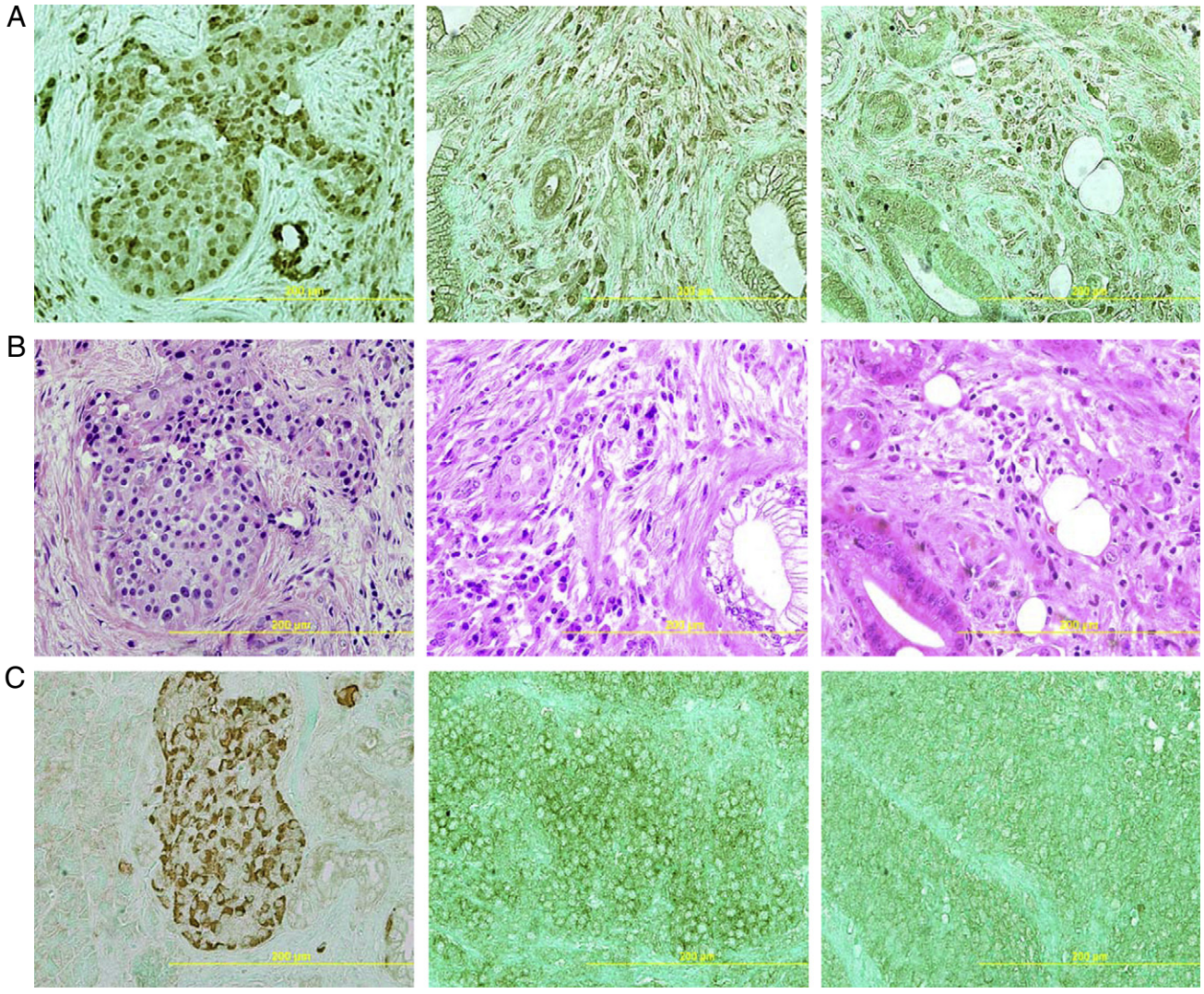

Fig. 5 – (A) Immunohistochemistry staining of PEDF in 3 pancreas cancer patients #44, 47, 101, (B) Hematoxylin/Eosin staining of same regions as PEDF slides. Nuclei are stained dark purple (C) PEDF staining of: (left to right) Non-tumor Pancreas tissue indicating cytoplasmic staining, Brain and Prostate Cancer tissues (all are 400× magnification).

therapeutic targets [28–33]. In more global proteomic approaches (i.e. using isotope labeling prior to LC/MS analysis) there have been as many as 770 proteins identified, with only a fraction up-regulated [34–37]. By contrast we believe the workflow reported here, with nearly 2400 proteins identified and over 100 showing statistically significant changes in expression, represents an efficient, straightforward and statistically guided approach to global profiling of the pancreatic cancer proteome.

The comprehensive nature of our proteomic profiling supports the work done by other groups which demonstrate that initial screening of tissues for identification of potential proteins of interest can be facilitated by utilizing subcellular fractionation as an initial means of sample separation [38–41]. The primary benefits are the time- and labor-efficient sample preparation and the additional protein localization information which is gained by employing the fractionation strategy. An additional benefit is the usefulness of the detergent based strategy for analysis of frozen tissue specimens. Freeze-thaw cycles can potentially disrupt the organellar membranes, resulting in loss of more traditional membrane based means of subcellular fractionation. By using differential detergent extractions we also eliminate the need for ultracentrifugation as these protocols can be carried out in a bench top centrifuge.

The validity of our data is strengthened by the fact that all of the proteins chosen for validation have been previously published in connection with pancreatic cancer. Both Biglycan and Thrombospondin-2 have been associated with increased expression in pancreatic cancer [26,42–44]. Biglycan is a component of the extracellular matrix and is known to be a target of TGF-β in pancreatic cancer cell lines [42]. Expression of Thrombospondin-2 by pancreatic stellate cells has been shown to promote pancreatic cancer invasion in vitro [45]. As components of the tumor microenvironment these two proteins can be considered as biomarkers with particular importance in pancreatic cancer due to the significant role of fibrosis in the pathogenesis of this disease [46]. βIGH3 is also involved in TGF-β

signaling and has been identified in pancreatic cancer[20] as well as in lung and colorectal carcinomas [47].

A putative nuclear translocation signal has been identified within the amino acid sequence of PEDF [48]. In addition, PEDF has been identified as an inhibitor of caspase independent apoptosis, which is triggered by AIF translocation to the nucleus and is interrupted by PEDF up-regulation of Bcl-2 [49]. Further study is needed to determine whether the increase in PEDF expression and nuclear localization in pancreatic cancer is a function of this apoptosis-inhibitory pathway.

While PEDF, βIGH3 and Thrombospondin-2 have all been identified in the human plasma proteome [24,25], all four of the proteins of interest have been reported as being secreted, [26] making each of them a potential target for validation of minimally invasive diagnostic value. More in depth investigation is in progress to determine the sensitivity and specificity with which any or all might be utilized in this manner. Furthermore, we are working with serum and urine to develop sensitive and specific absolute quantification techniques for each of these markers using additional technology, including selected reaction monitoring by triple quadrupole mass spectrometry with the goal of a large scale validation study.

Overall, we believe that the use of subcellular fractionation in conjunction with gel electrophoresis and LC-mass spectrometric analysis is a powerful screening tool for the initial identification of potential biomarkers of disease.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jprot.2010.08.006.

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