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Reverse-phase protein array analysis to identify biomarker proteins in human pancreatic cancer

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Abstract

Background—Pancreatic cancer is the fourth leading cause of cancer death in the U.S. The high mortality rate of patients with pancreatic cancer is primarily due to the difficulty of early diagnosis and a lack of effective therapies. There is an urgent need to discover novel molecular targets for early diagnosis and new therapeutic approaches to improve the clinical outcome of this deadly disease.

Aim—we utilized the reverse-phase protein assay (RPPA) to identify differentially expressed biomarker proteins in tumors and matched adjacent, normal-appearing tissue samples from 15 pancreatic cancer patients.

Methods—The antibody panel used for the RPPA included 130 key proteins involved in various cancer-related pathways. The paired *t* test was used to determine the significant differences between matched pairs, and the false discovery rate-adjusted *p* values were calculated to take into account the effect of multiple comparisons.

Results—After correcting for multiple comparisons, we found 19 proteins that had statistically significant differences in expression between matched pairs. However, only four (AKT, β -catenin, GAB2, and PAI-1) of them met the conservative criteria (both a *q* value <0.05 and a fold-change of $\geq 3/2$ or $\leq 2/3$) to be considered differentially expressed. Overexpression of AKT, β -catenin, and GAB2 in pancreatic cancer tissues identified by RPPA has also been further confirmed by western blot analysis. Further analysis identified several significantly associated canonical pathways and overrepresented network functions.

Conclusion—GAB2, a newly identified protein in pancreatic cancer, may provide additional insight into this cancer's pathogenesis. Future studies in a larger population are warranted to further confirm our results.

Keywords

pancreatic cancer; reverse-phase protein arrays; proteomic profiling; biomarkers

Introduction

Pancreatic cancer is the fourth leading cause of cancer-related mortality in the United States, with an estimated 42,920 new cases and 37,390 deaths in 2012 [1]. Pancreatic cancer has an extremely poor prognosis, with a 5-year relative survival rate of less than 4% associated with all cancer stages [1,2]. The high mortality rate of patients with pancreatic cancer is primarily due to late-stage diagnosis and a lack of effective treatment. In the majority of these patients the cancer is diagnosed at the locally advanced stage or when distant metastasis has already occurred, as early stage pancreatic cancer is generally asymptomatic. Unfortunately, only 10%–20% of patients with pancreatic cancer are considered eligible for surgical resection at the time of diagnosis [3]. Single-agent gemcitabine chemotherapy, the current standard first-line treatment for advanced or metastatic pancreatic cancer, has only shown modest efficacy, with response rates of less than 10% and a median survival duration of about 6 months [4,5]. Although numerous attempts have been made during the past decades to identify potential biomarkers for pancreatic cancer [6,7], no molecular biomarkers with adequate diagnostic or prognostic values have been identified [8]. Cancer antigen 19-9 has been the most extensively studied and commonly used tumor marker for pancreatic cancer, but it has failed to show satisfactory sensitivity and specificity for early detection and diagnosis [9,10]. There is thus an urgent need to discover novel molecular targets for early diagnosis and new therapeutic approaches to improve the clinical outcome of this deadly disease.

Many high-throughput analytical platforms (including genomic, proteomic, transcriptomic, and metabolomic strategies) have been used to identify candidate biomarkers and new drug targets for cancer. Although gene expression profile studies can be informative, mRNA levels do not necessarily correlate with protein expression or disease progress [11,12]. Cancer biomarker research that identifies differentially expressed proteins has become increasingly popular not only because the majority of current drug targets are proteins [13] but also because proteomic approaches can characterize posttranslational protein modifications involved in cancer progression [14,15].

Recent advances in proteomic techniques, including RPPA, are beginning to shed light on the importance of different proteins and their posttranslational modification in tumorigenesis. Therefore, we used reverse-phase protein assay (RPPA), a functional proteomic approach, to profile differentially expressed proteins between samples of human pancreatic cancer and matched adjacent, normal-appearing tissue. Finding novel proteins associated with pancreatic cancer could unravel the molecular events of this lethal disease and may help identify diagnostic biomarkers and therapeutic strategies, as well as provide insight into its pathogenesis.

Materials and Methods

Materials

Pancreatic primary tumor samples and matched adjacent, normal-appearing samples were collected from a total of 15 patients who had had no prior therapy and who had undergone

surgical resection for pancreatic cancer at The University of Texas MD Anderson Cancer Center. All tissues were obtained immediately after surgical resection and snap-frozen in liquid nitrogen and stored at -80°C until protein were extracted. The current study was approved by and conducted in accordance with the policies of the Institutional Review Board at MD Anderson Cancer Center.

Protein Extraction

For our analysis, we cut the tissue samples into small pieces, stored them on dry ice, and then homogenized them with ice-cold radioimmunoprecipitation assay (RIPA) buffer (#9806, Cell Signaling Technology, Danvers, MA, USA) that contained fresh protease/phosphatase inhibitors (#5872, Cell Signaling Technology). We set the samples on ice during homogenization to prevent heating. The lysate was centrifuged for 10 minutes at $14,000 \times g$ in a cold microfuge, and the supernatant was collected for RPPA analysis. Protein concentrations were determined using bicinchoninic acid protein assay reagents (Thermo Scientific/Pierce, Rockford, IL, USA).

RPPA

RPPA is a high-throughput method that can concurrently test hundreds of different proteins and pathways at different expression levels and posttranslational modifications, including cleavage and activated phosphorylated states. In this study, the RPPA assay was performed at the Functional Proteomics RPPA Core Facility at MD Anderson, as previously described [16]. Briefly, serial diluted protein lysates were arrayed onto nitrocellulose-coated slides (Grace Bio-labs, Bend, OR, USA) using an Aushon 2470 Arrayer (Aushon BioSystems, Billerica, MA, USA), including the spots corresponding to positive and negative controls prepared from mixed-cell lysates and dilution buffer, respectively. Each slide was probed with a validated primary antibody plus a biotin-conjugated secondary antibody. The binding signal obtained was amplified using a Catalyzed Signal Amplification System kit (#K1500, Dako, Carpinteria, CA, USA) and visualized by a diaminobenzidine colorimetric reaction. To generate spot intensity, we used customized software (Microvigene, VigeneTech, Carlisle, MA, USA) to scan, analyze, and quantify the images of stained slides. Each dilution curve was fitted with a logistic model ("Supercurve Fitting," developed by the Department of Bioinformatics and Computational Biology at MD Anderson Cancer Center, Houston, TX, USA [17]). All the data were normalized for protein-loading correction factor and transformed to linear values.

Antibodies for RPPA

A panel of 130 antibodies detecting total protein and/or its activated forms (such as phosphorylated or cleaved proteins) was included in this RPPA analysis (Appendix will be provided upon request). The antibody panel reacted with key proteins involved in critical signaling pathways and networks, such as the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway, the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway, the tyrosine kinase pathway, cell death and survival, cell-cycle regulation, cell growth and proliferation, and cellular movement.

Western Blot Analysis

Western blot analysis was performed by standard methods. Briefly, 25 ug of protein were separated by SDS-PAGE gel, transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were incubated with blocking solution [5% nonfat milk in TBS with 0.1% Tween 20 (pH 7.5)] for 1 h at room temperature, and then blotted with relevant antibodies at 4 degree overnight. HRP-conjugated secondary antibodies were detected by using the Enhanced Chemiluminescence kit (Amersham Biosciences, Piscataway, NJ, USA). Antibodies for GAB2 and PAI-1 were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies for AKT and β -catenin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-GAPDH antibody (Abcam, Cambridge, UK) was used as an internal control to visualize the total protein loading.

Cluster and Pathway/Network Analysis of Differentially Expressed Genes

We performed unsupervised and supervised hierarchical clustering analysis of differentially expressed genes and used open source Cluster 3.0 software [18] to classify the samples into statistically similar groups, and the resulting heat maps were visualized in TreeView 1.6 [18]. We uploaded the list of differentially expressed proteins into the Ingenuity Pathways Analysis (IPA) software (Ingenuity Systems, Redwood City, CA, USA; <http://www.ingenuity.com/>) to annotate significant molecular functions, canonical pathways, and gene network analysis. IPA was also used to construct and visualize molecular interaction networks.

Statistical Analysis

The paired *t* test was used to determine the significance of the difference in protein expression between matched pancreatic cancer samples and adjacent, normal-appearing tissue samples. To take into account the effect of multiple comparisons, we calculated the false discovery rate (FDR)-adjusted *p* values using the Benjamini-Hochberg method. All statistical analysis used the SAS program (SAS/STAT version 9.1.3; SAS Institute, Cary, NC, USA), and a FDR cutoff of <0.05 (*q* value) was applied as the statistical significance threshold.

Results

Proteomic Markers Differentiate Pancreatic Cancer from Adjacent Normal-Appearing Tissue

Our study population comprised 12 women and three men, aged between 48 and 80 years (mean $\pm$ standard deviation: 64.3 $\pm$ 8.8 years, median: 66.5 years). Protein expression data from 15 pairs of resected pancreatic cancer specimens and corresponding adjacent, normal-appearing tissue were quantified by RPPA. Clustering analysis demonstrated a distinct variation/division in expression between pancreatic cancer specimens and adjacent, normal-appearing tissue specimens (Fig. 1).

Of the 130 proteins we analyzed, the expression differences of 19 proteins between the matched pancreatic cancer samples and normal-appearing tissue samples remained

statistically significant after correction for multiple testing (FDR-adjusted p value, or $q < 0.05$): AKT, BCL2L1, BCL2L11, BRAF, CHEK1, CTNNB1, FGFR1, FRAP1, GAB2, IRS1, NCOA3, RB1, RPS6KB1, SERPINE1, TSC2, WWTR1, YAP1 (both the total and phosphorylated protein at Ser127), and YWHAE (Table 1). However, only AKT, beta(β)-catenin, GAB2, and PAI-1 met the conservative criteria (both a q value < 0.05 and a median fold-change either $\geq 3/2$ or $\leq 2/3$ between matched pairs) to be considered differentially expressed between paired normal tissue and cancer samples in this study (range, 1.6–2.0; see Table 1).

Western Blot Analysis

Next, we used the same protein lysates used in RPPA to validate the differential expression of AKT, β -catenin, GAB2, and PAI-1 by western blot analysis. Among those 15 pairs of pancreatic cancer and adjacent normal-appearing samples, four pairs were randomly selected for western blot. As shown in Fig. 2, GAB2, AKT and β -catenin were highly overexpressed in every pancreatic cancer sample compared to its matched normal tissue. These results were clearly consistent with the higher level of expression of GAB2, AKT and β -catenin respectively in cancer sample from RPPA analysis (Table 1 and Fig. 1). However, higher level of PAI-1 expression was only observed in one cancer sample compared to its matched normal tissue.

Pathway and Network Analysis

To identify the most significantly associated canonical pathways and overrepresented molecular network functions, we further analyzed a selected gene list of 19 tumor-associated statistically significant proteins ($q < 0.05$) using IPA software. The proteins we identified can be categorized according to their functions. Table 2 illustrates the top three network functions (with their IPA scores), related diseases and disorders, and associated molecular and cellular functions (along with p values and the number of molecules involved) representing key genes.

As shown in Fig. 3, the top five canonical pathways identified from proteins differentially expressed are PI3K/AKT signaling ($p = 4.33 \times 10^{-15}$), molecular mechanisms of cancer ($p = 8.4 \times 10^{-13}$), ovarian cancer signaling ($p = 7.95 \times 10^{-11}$), glioblastoma multiforme signaling ($p = 1.62 \times 10^{-10}$), and p53 signaling ($p = 6.71 \times 10^{-10}$). Specifically, we found that eight significant proteins (AKT, BCL2L1, CTNNB1, GAB2, MTOR, RPS6KB1, TSC2, and YWHAE) were involved in the PI3K/AKT signaling pathway; nine significant proteins (AKT, BCL2L1, BCL2L11, BRAF, CHEK1, CTNNB1, GAB2, IRS1, and RB1) were part of the molecular mechanisms of cancer pathway; and six significant proteins (AKT, BRAF, CTNNB1, MTOR, RB1, and RPS6KB1) were involved in the ovarian cancer signaling pathway. Fig. 4 demonstrates the global molecular network of four differentially expressed proteins (AKT, β -catenin, GAB2, and PAI-1, defined as having both $q < 0.05$ and an average fold-change either $\geq 3/2$ or $\leq 2/3$ between matched pairs) that were identified by the Ingenuity Pathways Knowledge Base. Both of direct and indirect relationships are included in this diagram.

Discussion

In this study, we used proteomic analysis to evaluate the protein expression pattern in pancreatic cancer tissues and matched adjacent, normal-appearing tissues. Among the 19 proteins that showed a statistically significant difference between these samples ($q < 0.05$), four proteins (phosphorylated FGFR1 at Thr766, phosphorylated CHK1 at Ser345, phosphorylated TAZ at Ser89, and 14-3-3 epsilon) had significantly lower levels in pancreatic tissue than in the matched adjacent tissue; the other 15 proteins had significantly higher levels in pancreatic cancer tissue than in the matched normal tissues (Table 1). The four differentially expressed proteins (AKT, β -catenin, GAB2, and PAI-1) we identified by RPPA are involved in the functional network of tumor morphology and cellular function and maintenance. The altered expression of the genes *AKT*, *β -catenin*, and *PAI-1* has previously been associated with pancreatic cancer in gene expression studies [19–22]. The overexpression of AKT, β -catenin, and PAI-1 in pancreatic cancer observed in RPPA confirms prior findings on the public database of the National Center for Biotechnology Information Gene Expression Omnibus [23]. However, when we used western blot to validate our RPPA result, only AKT, β -catenin, and GAB2 showed a distinct correlation between western blot and RPPA. To our knowledge, our study present here is the first report of GAB2 overexpression in pancreatic cancer tissue.

Pancreatic cancer is genetically complex, with individual tumors averaging more than 60 different genetic alterations [24]. Key genes mutated at high frequency in most pancreatic cancer include *KRAS2*, *CDKN2A/p16*, *TP53*, and *SMAD4/DPC4* [24,25]. Many additional genetic mutations and molecular alterations are linked to the development and/or progression of pancreatic cancer. Despite the gene expression aberrations and genetic mutations that may underlie many different types of genetic disease, many diseases are caused by the defective proteins [15]. Proteomic analysis has become a powerful tool in detecting and identifying candidate biomarker proteins or potential targets for therapy.

AKT (also known as protein kinase B) is an evolutionarily conserved serine/threonine kinase. The AKT kinase is a major downstream target of the PI3-K pathway that is activated by growth factors, cytokines and insulin. AKT is emerging as a key player in human cancers, ranging from gastric and ovarian cancers to estrogen receptor-negative breast cancer and androgen-insensitive prostate cancer [20,26,27]. In addition, overexpression and/or hyperactivation of AKT in tumor cells have caused resistance to agents that target elements upstream of AKT [28,29]. Once activated, AKT regulates multiple cellular functions, including survival, proliferation, growth, and various aspects of metabolism [26,30–33]. Phosphorylation of Thr-308 of AKT by PI-3K is required for AKT activation, while Ser-473 phosphorylation is only needed for maximal activity. In our study, we observed that the pancreatic cancer samples had higher levels of the total protein of AKT than did the matched adjacent, normal-appearing tissue samples. However, none of AKT's active forms, such as phosphorylated AKT at Thr308 or Ser473, were significantly overexpressed in pancreatic cancer tissue ($q > 0.05$, data not shown). In the same pathway, the total protein of AKT's downstream molecules, such as TSC2, mTOR, and p70S6K, also had significantly higher levels in the pancreatic cancer samples, but not in the normal-appearing tissue; however, the active forms of these proteins, including phosphorylated

TSC2 at T1462 (which is phosphorylated by AKT) and phosphorylated p70S6K at S371 and T389, showed no difference, respectively ($q>0.05$, data not shown).

GAB2 is a scaffolding protein that contains various structural domains and docking sites and serves as an adapter protein that acts upon downstream membrane receptors, including cytokine, antigen, hormone, cell matrix and growth factor receptors, to regulate multiple signaling pathways involved in cell proliferation, migration, and differentiation [34–38]. GAB2 is essential for two major signaling pathways in cancer, the PI3-AKT and the ERK signaling pathways, and thus GAB2 regulates a number of key cellular processes [39,40]. In addition to its important role in the signaling transduction pathway, GAB2's role in cancer has been recently emerging. GAB2 overexpression has been observed in various malignancies such as breast cancer [41,42], metastatic melanoma [43], acute myeloid leukemia [44], ovarian cancer [45], gastric cancer [46], and lung cancer [47], suggesting its function as an oncogene. In our study, we observed for the first time, to our knowledge, that pancreatic cancer tissue had higher levels of GAB2 than did matched normal-appearing tissue. Considering the emerging role of GAB2 in cancer, our results suggest that GAB2 could be involved in pancreatic cancer tumorigenesis and could serve as a novel prognostic and therapeutic biomarker for pancreatic cancer.

β -catenin, encoded by *CTNNB1*, is part of a complex of proteins that constitute the adherens junction and is an integral part of the canonical Wnt signaling pathway. The Wnt/ β -catenin signaling pathway plays a pivotal role in regulating cellular processes involved in development, differentiation, and adult tissue homeostasis [48]. Aberrant Wnt/ β -catenin signaling is widely implicated in cancers of the gastrointestinal tract, including pancreatic cancer [19]. Alterations in β -catenin expression are common in pancreatic cancer; one study showed that more than 65% of pancreatic tumors had increased levels of total β -catenin, consistent with observations of enhanced membranous, cytoplasmic, and nuclear localization of β -catenin [49]. In addition, the direct inhibition Wnt/ β -catenin signaling by dominant-negative lymphoid enhancer-binding factor or siRNA/shRNA knockdown of β -catenin suppressed human pancreatic cancer cell line growth and survival in vitro [50,51]. Our observation of the overexpression of β -catenin in pancreatic cancer supports an oncogenic role of β -catenin in pancreatic cancer.

PAI-1, encoded by *SERPINE1*, is a member of the serpin family of serine protease inhibitors and is one of the key regulators of tumor invasion and metastasis [52,53]. Overexpression of PAI-1 has been observed in oral squamous cell carcinoma, identified as a potential marker of initial invasion [54], and correlated with a poor prognosis [54,55]. Overexpression of PAI-1 has also been associated with cervical carcinoma [56]. In addition, one study found high levels of PAI-1 in pancreatic cancer via immunohistochemical staining [21], and another study used a gene expression array to detect high levels of PAI-1 in an orthotopic pancreatic tumor model in severe combined immunodeficiency mice [22]. Our study's observation of overexpression of PAI-1 in pancreatic cancer samples further supports PAI-1's role in pancreatic tumorigenesis.

Although our findings support the results of previous studies, our study had some limitations. First, our sample size was relatively small (15 pairs). However, our pilot study

shed light on the protein expression profiles of pancreatic cancer, which has not been examined by RPPA previously. Second, although RPPA can provide posttranslational modification information such as phosphorylation and cleavage of protein, the panel of antibodies used in this RPPA mainly focused on the key proteins involved in several signaling pathways, such as cell death and survival, cellular growth and proliferation, cellular development, cell-cycle regulation, and cellular movement. Because our antibodies were limited, we could not provide a whole-spectrum protein expression profile. Third, adjacent, normal-appearing tissue from cancer patients might nevertheless carry some early transformations or be contaminated with cancer cells, which could bias analysis. Further confirmation with a larger sample size and a wider selection of antibodies will be more informative, and validation of our observations using a different methodology, such as Western blot analysis and/or immunohistochemistry, is necessary.

In conclusion, we observed that pancreatic cancer samples have different protein expression profiles than do samples from adjacent, normal-appearing tissue. Understanding protein expression in normal-appearing tissue is vital to understanding the expression changes in cancer. Among the four differentially expressed proteins we identified, GAB2 has not been reported previously. Our results likely have important implications for diagnostic and therapeutic strategies for patients with pancreatic cancer.

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Fig. 1.

The heat map of our RPPA data from pancreatic cancer samples and adjacent, normal-appearing tissue (n=15). The horizontal axis shows the samples tested in the RPPA and the vertical axis lists 19 proteins that had statistically significant differences in expression ($q < 0.05$). Abbreviations: RPPA, reverse-phase protein array

Fig. 2.

Western blot analysis of four RPPA-identified proteins in the pancreatic tissues. Protein lysates from four independent pancreatic cancer samples (lanes T1–T4) and their matched adjacent, normal-appearing tissues (lanes N1–N4) were used. GAPDH antibody was used as a loading control.

Fig. 3.

Top five canonical pathways affected by the 19 proteins that reached statistical significance for differential expression, identified by IPA

Fig. 4.

Pathway analysis and molecular interactions of focus genes *AKT*, *CTNNB1*, *GAB2*, and *SERPINE1* (highlighted in pink). Arrows indicate the direction of signaling. The signaling pathway model was made with Ingenuity Pathway Analysis software

Table 1

Protein expression differences between matched pancreatic cancer samples and adjacent, normal-appearing tissue from 15 patients

Protein ID	Gene	Fold change* (T/N, median)	p value	q value
FGFR1pT766	<i>FGFR1</i>	0.80	.0004	.0152
CHK1pS345	<i>CHEK1</i>	0.84	.0003	.0152
TAZpS89	<i>WWTR1</i>	0.84	.0016	.0202
14-3-3 epsilon	<i>YWHAE</i>	0.88	.0021	.0207
YAP	<i>YAP1</i>	1.16	.0013	.0191
TSC2	<i>TSC2</i>	1.17	.0028	.0234
Bcl-X	<i>BCL2L1</i>	1.19	.0019	.0202
AIB1	<i>NCOA3</i>	1.20	.0029	.0234
mTOR	<i>FRAP1</i>	1.21	.0012	.0191
IRS1	<i>IRS1</i>	1.28	.0012	.0191
BRAF	<i>BRAF</i>	1.30	.0013	.0191
BIM	<i>BCL2L11</i>	1.34	.0064	.0462
p70S6K	<i>RPS6KB1</i>	1.36	.0011	.0191
YAPpS127	<i>YAP1</i>	1.36	.0002	.0152
RbpS807	<i>RB1</i>	1.39	.0026	.0234
GAB2	GAB2	1.62	.0046	.0348
AKT	AKT	1.69	.0005	.0167
β-catenin	CTNNB1	1.75	.0019	.0202
PAI-1	SERPINE1	1.97	.0072	.0493

* Ratio of spot intensity between matched pancreatic cancer samples and adjacent, normal-appearing tissue. Four differentially expressed proteins (met the criteria of both a q value <0.05 and a median fold-change either $\geq 3/2$ or $\leq 2/3$ between matched pairs) are highlighted in bold.

Table 2

Top associated network functions and bio functions of the 19 proteins that showed a statistically significant difference, identified by IPA

Associated network functions	Score
Cell Morphology, Cellular Function and Maintenance, Cell Death and Survival	22
Cell Death and Survival, Tumor Morphology, Cancer	11
Cancer, Organismal Injury and Abnormalities, Endocrine System Disorders	10

Disease and disorders	<i>p</i> value	# Molecules
Cancer	1.09E-12 - 4.78E-04	19
Hematological Disease	3.20E-11 - 2.82E-04	12
Dermatological Diseases and Conditions	9.44E-09 - 1.05E-04	10
Neurological Disease	1.24E-08 - 2.28E-04	10
Endocrine System Disorders	1.92E-08 - 3.79E-04	15

Molecular and cellular functions	<i>p</i> value	# Molecules
Cell Death and Survival	6.30E-14 - 4.63E-04	18
Cellular Development	8.29E-14 - 4.43E-04	18
Cellular Growth and Proliferation	8.29E-14 - 4.94E-04	18
Cell Morphology	1.32E-13 - 2.88E-04	18
Cellular Function and Maintenance	7.14E-13 - 3.48E-04	16

Abbreviations: IPA, Ingenuity Pathway Analysis.