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Phosphoproteomic Analysis of Pancreatic Ductal Adenocarcinoma Cells Reveals Differential Phosphorylation of Cell Adhesion, Cell Junction and Structural Proteins

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Abstract

In this present work, we characterized the phosphoproteomes of pancreatic ductal adenocarcinoma (PDAC) cells and normal pancreatic duct cells by mass spectrometry using LTQ-Orbitrap. We identified more than 700 phosphoproteins from each sample, and revealed differential phosphorylation of many proteins involved in cell adhesion, cell junction, and cytoskeleton. Since post-translational phosphorylation is a common and important mechanism of acute and reversible regulation of protein function in mammalian cells, an understanding of differential phosphorylation of these proteins and resulting signal transduction changes in PDAC will help in comprehending the complex dynamics of tumor invasion and metastasis in pancreatic cancer.

Keywords: Mass spectrometry; LTQ-Orbitrap; phosphoproteomics; Pancreatic ductal adenocarcinoma; Cell adhesion; Cell junction; Cytoskeleton

Introduction

Pancreatic cancer is the fourth leading cause of cancer death in the United States and Europe. The absence of early symptoms or clinical-pathological markers results in diagnosis at a late, inoperable stage in more than 80% of cases. Most patients die within 12 months and only 4% survive for 5 years after diagnosis [1,2]. In the past decade, various mass spectrometry-based approaches have been applied to investigate the proteomes of diseased and normal samples from pancreatic tissues, juice, cell lines, and serum, with the goals of dissecting the abnormal signaling pathways underlying oncogenesis and identifying new biomarkers [3-13]; however, the description of the content of phosphoproteins in pancreatic cancer cells were limited. Since this information archive represents many of the new drug targets for kinase-based inhibitors, it is of critical importance to characterize this molecular information source.

Post-translational phosphorylation is a common and important mechanism of acute and reversible regulation of protein function in mammalian cells, and largely controls cellular signaling events that orchestrate biological functions. In humans, approximately 2% of the genome codes for kinases and phosphatases (there are ~500 kinases and 100 phosphatases in humans) [14]. A large number of oncogene products have protein kinase activity and are themselves substrates for protein kinases and phosphatases. Studies of mammalian cells metabolically labeled with [^{32}P]orthophosphate suggest that as many as one-third of all cellular proteins are covalently modified by phosphorylation [15,16]. At physiological p^{H}, the side chains of Ser/Thr/Tyr are not charged, and phosphorylation of Ser/Thr/Tyr will introduce negative charge to these amino acid residues. Consequently, protein phosphorylation can affect catalytic activity, localization of a protein in the cell, protein stability, and the ability of a protein to dimerize or form a stable complex with other molecules. Protein phosphorylation and dephosphorylation function together in signal transduction pathways to induce rapid changes in response to hormones, growth factors, and neurotransmitters [17]. Thus, global characterization of the phosphoproteome is essential for a systematic understanding of cellular behavior of pancreatic cancer cells.

Today, high sensitive reversed-phase liquid chromatography coupled nanospray tandem mass spectrometry (LC-MS/MS) is the most commonly used technique for large-scale protein identification and global profiling of post-translational modifications from complex biological mixtures [18-21]. Due to the low stoichiometry of most phosphorylated proteins, enrichment of phosphopeptides by immobilized metal ion affinity chromatography (IMAC), or titanium dioxide (TiO_2) chromatography, is advantageous or required before MS analysis in order to detect and measure such low abundance analytes [22]. In an effort to systematically reveal phosphoproteins in pancreatic cancer, particularly focusing on the proteins involved in cell adhesion, cell junction, and cytoskeleton, we performed phosphoproteomic analysis of TiO_2-enriched phosphopeptides from PDAC cells and normal pancreatic duct cells by LC-MS/MS, and identified a large number of proteins with differential phosphorylation, which can serve as a launch-point for further exploration and analysis to understand tumor invasion and metastasis.

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Materials and Methods

Sample preparation

CFPAC-1 cells (metastatic cell line derived from PDAC patients, ECACC ref. No: 91112501) were cultured at 37°C in Dulbecco modified Eagle's medium (DMEM) (Invitrogen) supplemented with 20 mM glutamine, 10% fetal calf serum (FCS), and 40 µg/mL Gentamycin with humidified 5% CO_2. The cells were harvested and washed with Hank's balanced salt solution (Sigma-Aldrich). The cell pellet was freeze-dried overnight and stored at -80°C until use. Normal human pancreatic duct cells were obtained by primary culture of pancreatic duct from a single brain death donor under IRB approval (San Raffaele Scientific Institute, Italy) based on the published method [23]. Through a period of suspension culture, epithelial cells were enriched while stromal components were reduced to less than 1%, confirmed by FACS analysis with markers for epithelial (ESA, Ca19.9) and fibroblast (CD73, CD105, CD90) phenotype. The CFPAC-1 cells and normal duct cells were resuspended for 1 hour in lysis buffer consisting of Tris/HCl (50mM, p^H 7.4), NaCl (150 mM), Triton X-100 (0.5% w/v), NP-40 (0.5% w/v), 80 mM dithiothreitol (DTT), 10 µL/mL protease inhibitor cocktails (Sigma-Aldrich), 1 mM PMSF, 1 mM Na_3VO_4 and PhosStop phosphatase inhibitor cocktail (Roche), sonicated for 30 s, and centrifuged at 16,000× g for 10 min. The supernatants were precipitated with 4 volume of acetone (Sigma-Aldrich) overnight at -20°C and centrifuged at 9,000× g for 5 min. The pellets were dried by lyophylization (Heto, Dry Winner) for 2 hours.

Trypsin digestion and desalting

The cell pellets were resuspended in 200 µL of 8 M urea, and the protein concentration was measured by Bradford Assay (BioRad). The proteins were transferred to a 1.5-mL eppendorf tube, reduced by 10 mM dithiothreitol (DTT) for 30 min at 37°C, and then alkylated by 50 mM iodoacetamide for 20 min at room temperature. The concentrated urea in the sample was diluted to a final concentration of 2 M, and the proteins were digested by trypsin at 37°C for 6 h in a buffer containing ammonium bicarbonate (50 mM, p^H 9). The digestion mixture was then acidified by adding glacial acetic acid to a final concentration of 2% and desalted by SepPak C_{18} column (Waters).

TiO_2 enrichment of phosphopeptides

Phosphopeptides were enriched from the desalted 1 mg tryptic peptides using TiO_2 column (200 µm × 2 cm) packed in-house [24]. 100 fmol of standard phosphopeptide angiotensin II phosphate (Ang II-Phos) was added to the SepPak-cleaned sample. The sample was then mixed with an equal volume of Loading Buffer (200 mg/mL DHB, 5% TFA, 80% acetonitrile), and loaded into the TiO_2 column using the Pressure Cell (Brechbühler Inc.) with flow rate of 3 µL/min. The column was washed by 200 µL of Wash Buffer 1 (40 mg/mL DHB, 2% TFA, 80% acetonitrile) and 2 × 200 µL of a second Wash Buffer 2 (2% TFA, 50% acetonitrile) to remove non-phosphopeptides. Phosphopeptides were eluted from the column with the Elution Buffer (5% ammonia solution). Ammonia in the eluate was removed by lyophilization (~3 min), and the sample was acidified by adding glacial acetic acid to a final concentration of 2%, and desalted by ZipTip (Millipore).

Mass spectrometry for phosphopeptides identification

The purified phosphopeptides were analyzed by LC-MS/MS using an LTQ-Orbitrap mass spectrometer (Thermo Fisher). LTQ-Orbitrap provides high accuracy mass measurement that is essential for the validation of modified peptide identifications and the reduction of false positive identifications. The reversed-phase LC column was slurry-packed in-house with 5 µm, 200 Å pore size C_{18} resin (Michrom BioResources, CA) in a 100 µm i.d. × 10 cm long piece of fused silica capillary (Polymicro Technologies, Phoenix, AZ) with a laser-pulled tip. After packing, the new column, the HPLC system (Surveyor MS Pump Plus from ThermoFisher) and the LTQ-Orbitrap, were tested by analyzing 100 fmol "Yeast Enolase Standard & Tryptic Digestion" from Michrom Bioresources, Inc. (catalogue number PTD/00001/46) to ensure that stable ESI, desired mass accuracy, peak resolution, peak intensity and retention time could be obtained. Additional iteration was performed to ensure reproducibility. 100 fmol of standard peptide angiotensin I (Ang I) were spiked into the sample as an internal standard. After sample injection, the column was washed for 5 min with mobile phase A (0.1% formic acid), and peptides were eluted using a linear gradient of 0% mobile phase B (0.1% formic acid, 80% acetonitrile) to 40% B in 120 min at 200 nL/min, then to 100% B in an additional 10 min. The HPLC gradient was shallower than that of general proteomic analysis since phosphopeptides are relative hydrophilic. Before and after analyzing one sample, the column was washed with HPLC mobile phase B for 30 min, then mobile phase A for 20 min at high flow rate (1 µL/min) to reduce potential carryover. The LTQ-Orbitrap mass spectrometer was operated in a data-dependent mode in which each full MS scan (60,000 resolving power) was followed by eight MS/MS scans where the eight most abundant molecular ions were dynamically selected and fragmented in by collision-induced dissociation (CID) using a normalized collision energy of 35%. The fragmented ions were detected by LTQ. The Dynamic Exclusion Time was 30 s, and the Dynamic Exculsion Size was 200. The "FT master scan preview mode", "Charge state screening", "Monoisotopic precursor selection", and "Charge state rejection" were enabled so that only the 1+, 2+, and 3+ ions were selected and fragmented by CID.

Mass spectrometry data analysis

Tandem mass spectra collected by Xcalibur (version 2.0.2) were searched against the NCBI human protein database (released in September 2009 with 37391 entries) using SEQUEST (Bioworks software from ThermoFisher, version 3.3.1) with full tryptic cleavage constraints, static cysteine alkylation by iodoacetamide, variable methionine oxidation, and variable phosphorylation of Ser/Thr/Tyr. Mass tolerance for precursor ions was 5 ppm and mass tolerance for fragment ions was 0.25 Da. The SEQUEST search results were filtered by criteria "Xcorr versus charge 1.8, 2.1, 3.0 for 1+, 2+, 3+ ions; ranked top #1; probability of randomized identification of peptide < 0.05". Confident peptide identifications were determined using these stringent filter criteria for database match scoring followed by manual evaluation of the phosphorylation site assignment. The "false discovery rate (FDR)" was estimated by searching a combined forward-reversed database as described by Elias [25].

Results and Discussion

The same amounts of proteins (1 mg) from CFPAC-1 cells and normal duct cells were digested by trypsin and the enriched phosphopeptides were identified by LC-MS/MS using LTQ-Orbitrap. Common MS normalization was done in order to reduce extraneous variability. Both exogenous control (spiking standard peptide Ang I and Ang II-Phos) and internal controls (phosphopeptides from protein AHNAK nucleoprotein isoform 1) were utilized to ensure that desired mass accuracy , peak intensity, peak retention time, and reproducible chromatography could be obtained from the two samples

(Figure 1). The SEQUEST search results were filtered by stringent criteria as described above and yielded 3011 mathced MS2 spectra from CFPAC-1 cells. Among these, 482 (16%) spectra were matched to non-phosphopeptides, and 2529 (84%) spectra were matched to phosphopeptides. A total of 1665 unique phosphopeptides was identified from 707 proteins with 1% FDR at phosphopeptide level. Similarly, the SEQUEST search results yielded 3076 matched MS2 spectra from normal duct cells. Among these, 523 (17%) spectra were matched to non-phosphopeptides, and 2553 (83%) spectra were matched to phosphopeptides. A total of 1572 unique phosphopeptides was identified from 736 proteins with 1% FDR at phosphopeptide level (Table 1). The MS result unveiled a large number of proteins with differential phosphorylation in CFPAC-1 and normal duct cells. Notably, many of these proteins are cell adhesion, cell junction and structural proteins.

Phosphorylation of cell adhesion proteins

Many cells bind to components of the extracellular matrix (ECM). Cell adhesion can occur either by focal adhesions, connecting the ECM to actin filaments of the cell, or by hemidesmosomes, connecting the ECM to intermediate filaments such as keratin. This cell-to-ECM adhesion is regulated by specific cell surface cellular adhesion molecules (CAMs) such as immunoglobulin superfamily (IgSF), integrins, cadherins, and selectins. The integrins, heterodimers composed of alpha and beta subunits, are a family of heterophilic calcium-independent CAMs that bind IgSF CAMs or the extracellular matrix; the cadherins, named for calcium-dependent adhesion, are a family of homophilic CAMs including cadherins, protocadherins, desmogleins, and desmocollins. The dynamic assembly and disassembly of focal adhesions plays a central role in cell migration [26].

Here, 19 unique phosphopeptides were identified from integrin beta 4 isoform 3 in CFPAC-1 cells, whereas only 4 of these phosphopeptides were found from the protein in normal duct cells (Table 2, Figure 2). Separately, differential phosphorylation was also observed in proteins of protocadherin 1 isoform 1, desmoglein 2, tensin, zyxin, PTPRF interacting protein alpha 1 isoform b, and secreted phosphoprotein 1 (also named osteopontin) isoform a (Table 2, Supplementary information Table 1S). Tesin is a multi-domain protein localized to focal

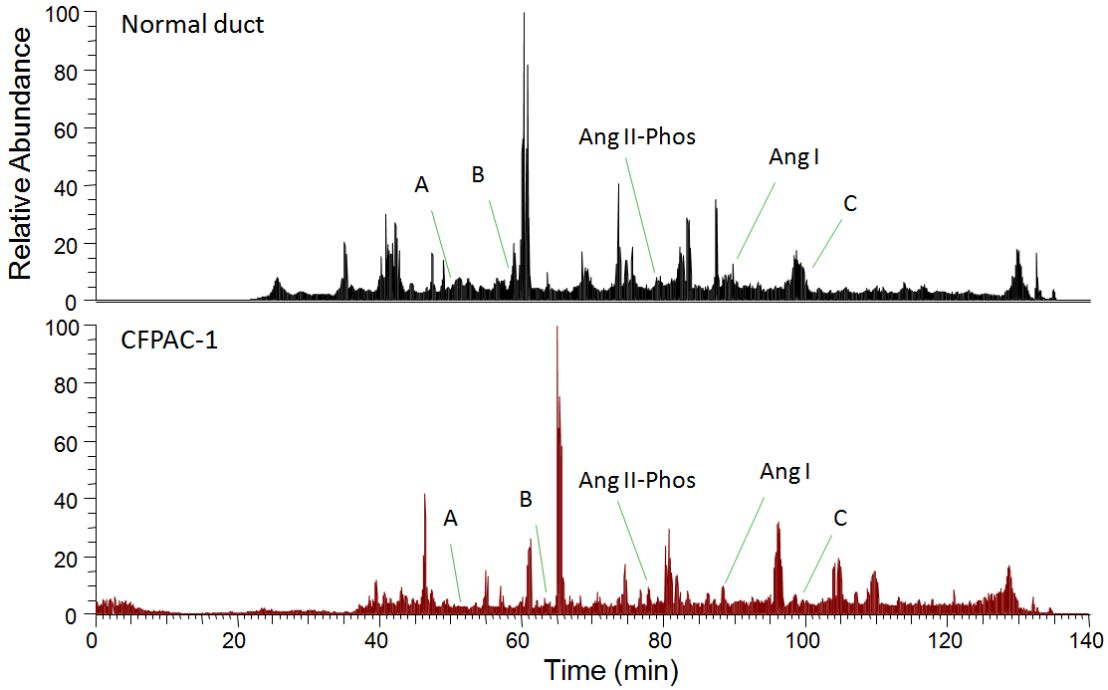

Figure 1: Chromatogram of LC-MS/MS analysis of TiO_2-enriched phosphopeptides from normal pancreatic duct and CFPAC-1 cells. Enriched phosphopeptides from each sample (1 mg) and 100 fmol standard peptide Ang I were loaded to C_{18} capillary column, and the eluted peptides were analyzed by LTQ-Orbitrap. The mass accuracy of Ang I and Ang II-Phos was within 5 ppm, and peak intensity of Ang I was ~ 1E8. For the normal duct sample, the retention time of exogenous control Ang II-Phos and Ang I were 78 min and 88 min, respectively, and the retention times of 3 internal control phosphopeptides (i.e. peak A, B, C) from AHNAK nucleoprotein isoform 1 were 50 min, 62 min and 98 min, respectively. Labeled peak A is the phosphopeptide LPSGSGAAS#PTGSAVDIR (2+ ion, m/z 861.9022); peak B is the phosphopeptide DIDISS#PEFK (2+ ion, m/z 615.7634); peak C is the phosphopeptide MYFPDVEFDIKS#PK (2+ ion, m/z 898.4021). These phosphopeptides were also identified in CFPAC-1 sample with similar retention times (± 2.0 min), indicating that the experimental procedure was reproducible and the MS results could be compared.

	Normal duct cells	CFPAC-1 cells
Total matched MS2 spectra	3076	3011
MS2 spectra matched to non-phosphopeptide (% total)	523 (17%)	482 (16%)
MS2 spectra matched to phosphopeptide (% total)	2553 (83%)	2529 (84%)
Total unique phosphopeptides	1572	1665
Total phosphoprotein	736	707

Table 1: Summary of phosphopeptide identification by LTQ-Orbitrap.

Protein and phosphopeptide	Functional annotation[a]	Accession number	Position[b]	Normal duct	CFPAC-1
integrin beta 4 isoform 3 precursor	cell adhesion	54607033			
LLELQEVDS#LLR			1061-1072	√	√
MDFAFPGS#TNSLHR			1377-1390	√	√
MDFAFPGSTNS#LHR			1377-1390		√
MDFAFPGS#TNS#LHR			1377-1390	√	√
MTTTSAAAYGTHLS#PHVPHR			1391-1410		√
VLS#TSSTLTR			1411-1420	√	√
VLSTSS#TLTR			1411-1420		√
VLS#TSS#TLTR			1411-1420		√
VLSTSS#T#LTR			1411-1420		√
VLS#TSS#T#LTR			1411-1420		√
DYNS#LTR			1421-1427		√
DYNS#LTRSEHS#HSTTLPR			1421-1438		√
DYSTLTSVSS#HDSR			1439-1452		√
DYSTLTSVS#S#HDSR			1439-1452		√
DYSTLTSVSSHDS#RLT#AGVPDTPTR			1439-1463		√
DYSTLTS#VSS#HDSRLT#AGVPDTPTR			1439-1463		√
LTAGVPDT#PTR			1453-1463		√
LTAGVPDTPT#R			1453-1463		√
HVT#QEFVSR			1725-1733		√
tensin	cell adhesion	66529407			
LLS#GFGLER			443-451	√	
SQS#FSEAEPQLPPAPVR			619-635	√	
S#GYIPSGHSLGTPEPAPR			764-781	√	
SGY#IPSGHSLGTPEPAPR			764-781	√	
EATSDPSRT#PEEEPLNLEGLVAHR			852-875	√	
EAFEEMEGTSPSS#PPPSGVR			951-970	√	
T#PTQPLLESGFR			1105-1116	√	
AQFSVAGVHTVPGS#PQAR			1164-1181	√	
TVGTNTPPS#PGFGWR			1184-1198	√	
VATTPGS#PSLGR			1263-1274	√	
HLGGSGSVVPGS#PCLDR			1303-1319	√	
QGS#PTPALPEK			1379-1389	√	
VSSPVASGMSSPSGGSTVS#FSHTLPDFSK			1411-1439	√	
VSS#PVASGMS#SPSGGSTVSFSHTLPDFSK			1411-1439	√	
VSS#PPPTIMQQNK			1503-1515	√	
zyxin	cell adhesion	4508047			
EKVSS#IDLEIDSLSSLLDDMTK			139-160	√	
FTPVASKFS#PGAPGGSGSQPNQK			273-295	√	
S#PGAPGPLTLK			344-354	√	
secreted phosphoprotein 1 isoform a	cell adhesion	91206462			
QNLLAPQNAVSS#EETNDFK			52-70	√	
QNLLAPQNAVS#SEET#NDFK			52-70	√	
AIPVAQDLNAPSDWDS#R			204-228	√	
FRIS#HELDSASSEVN			300-314	√	
FRIS#HELDSAS#SEVN			300-314	√	
catenin (cadherin-associated protein), beta 1	cell junction	4503131			
AAVSHWQQQS#YLDSGIHSGATTTAPSLSGK			20-49	√	
AAVMVHQLS#K			171-180	√	

	S#PQMVSAIVR			191-200	√	
	SPQMVS#AIVR			191-200	√	
	RTS#MGGTQQQFVEGVR			550-565	√	√
	RTSMGGT#QQQFVEGVR			550-565	√	
	RLS#VELTSSLFR			673-684	√	
	RLSVELTSS#LFR			673-684	√	
	RLS#VELTS#SLFR			673-684	√	
	RLS#VELTSS#LFR			673-684	√	
microtubule-associated protein 1B		stabilizing microtubules	153945728			
	SLMS#S#PEDLTK			828-838		√
	AAEAGGAEEQYGFLTT#PTK			1052-1070		√
	DVMSDETNNEETES#PSQEFVNITK			1141-1164		√
	VSPSKS#PSLSPSPPS#PLEK			1251-1269		√
	VSPSKSPSLS#PSPPS#PLEK			1251-1269		√
	VSPSKS#PSLS#PSPPS#PLEK			1251-1269		√
	SPSLSPSPPS#PLEK			1256-1269		√
	SVNFSLT#PNEIK			1276-1287		√
	VSAEAEVAPVS#PEVTQEVVEEHCASPEDK			1288-1316		√
	VSAEAEVAPVSPEVTQEVVEEHCAS#PEDK			1288-1316		√
	VSAEAEVAPVS#PEVTQEVVEEHCAS#PEDK			1288-1316		√
	TLEVVS#PSQSVTGSAGHTPYYQSPTDEK			1317-1344		√
	TLEVVS#PSQSVTGSAGHTPYYQS#PTDEK			1317-1344		√
	ASVS#PMDEPVPDSESPIEK			1375-1393		√
	ASVSPMDEPVPDSES#PIEK			1375-1393		√
	VLSPLRS#PPLIGSESAYESFLSADDK			1394-1419		√
	VLS#PLRS#PPLIGSESAYESFLSADDK			1394-1419		√
	VLSPLRS#PPLIGS#ESAYESFLSADDK			1394-1419		√
	QGS#PDQVS#PVSEMTSTSLYQDK			1436-1457		√
	KLGDVS#PTQIDVSQFGSFK			1496-1514		√
	VQSLEGEKLS#PK			1770-1781		√
	VQSLEGEKLS#PKS#DISPLT#PR			1770-1790		√
	SDIS#PLTPR			1782-1790		√
	SDIS#PLT#PR			1782-1790		√
	ESS#PLYSPTFSDSTSAVK			1791-1808		√
	ESSPLYS#PTFSDSTSAVK			1791-1808		√
	ES#SPLYS#PTFSDSTSAVK			1791-1808		√
	ESS#PLYS#PTFSDSTSAVK			1791-1808		√
	ESS#PLYS#PTFS#DSTSAVK			1791-1808		√
	TATCHSS#SSPPIDAASAEPYGFR			1811-1833		√
	S#PSDSGYSYETIGK			1915-1928		√
	TTS#PPEVSGYSYEK			1963-1976		√
	HMDPPPAPVQDRS#PS#PR			2197-2213		√
microtubule-associated protein 7		cytoskeleton	4505101			
	SVS#TMNLSK			181-189	√	
	LSSS#SATLLNSPDR			199-212	√	
	LLTPT#HSFLAR			229-239	√	
	LFVTPPEGS#SR			274-284	√	
	AAPAQVRPPS#PGNIRPVK			356-373	√	

[a] Functional annotation is based on Protein Knowledgebase at http://www.uniprot.org.
[b] Position of the initial and final peptide amino acids in the protein sequence.

Table 2: A partial list of identified proteins with differential phosphorylation in normal duct and CFPAC-1 cells.

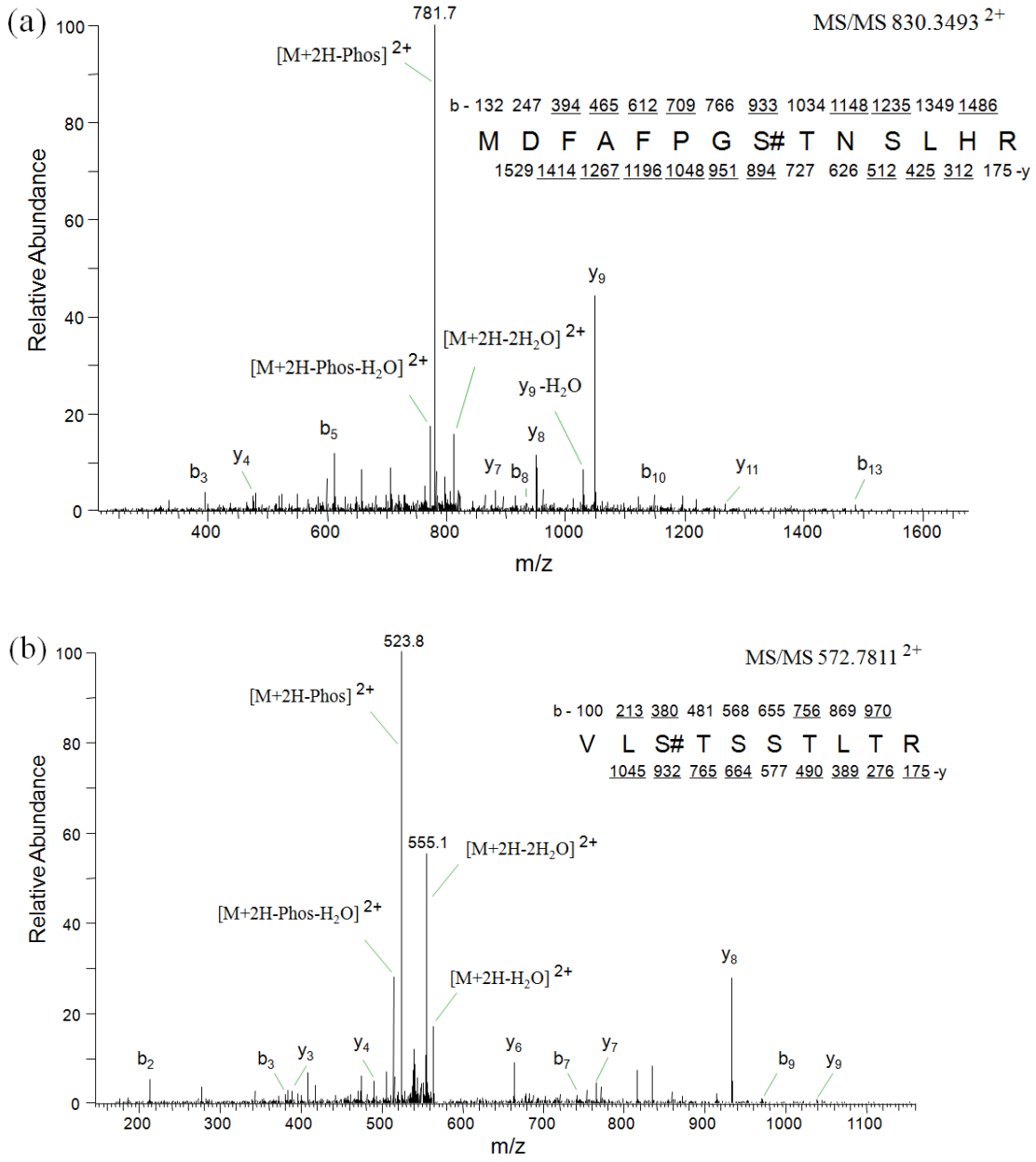

Figure 2: Identification of phosphoprotein integrin beta 4 isoform 3 by LC-MS/MS. Twenty-seven phosphospectra (corresponding to 19 unique phosphopeptides) were matched to integrin beta 4 isoform 3 in CFPAC-1 cells, and 6 (corresponding to 4 unique phosphopeptides) in normal duct cells. (a) Example CID spectrum of the phosphopeptide MDFAFPGS#TNSLHR (2+ ion m/z 830.3493) obtained from normal duct cells; (b) CID spectrum of the phosphopeptide VLS#TSSTLTR (2+ ion m/z 572.7811) obtained from CFPAC-1 cells. The spectrum is labeled to show singly-charged b and y ions, as well as ions corresponding to neutral loss of water and phosphate group.

adhesions. It links actin filaments to integrin receptors, and functions as a platform for assembly of signaling complexes at focal adhesions by recruiting tyrosin-phosphorylated molecules [27,28]. Zyxin is a zinc-binding phosphoprotein that concentrates at focal adhesions and along the actin cytoskeleton. Zyxin may function as a messenger in the signal transduction pathway that mediates adhesion-stimulated changes in gene expression and may modulate the cytoskeletal organization of actin bundles [29]. PTPRF interacting protein alpha 1 (short name of "Protein tyrosine phosphatase receptor type f polypeptide-interacting protein alpha-1"), also named Liprin-alpha-1, may regulate the disassembly of focal adhesions [30]. Osteopontin is an extracellular structural protein and an organic component of bone. It is also expressed in other tissues, and it is an integrin-binding phosphorylated glycoprotein, recognized as a key molecule in a multitude of biological processes such as bone mineralization, cell-matrix interaction, cancer metastasis, cell-mediated immune response, and inflammation [31,32].

Phosphorylation of cell junction proteins

Cell junctions are protein complexes that exist within the tissue of a multicellular organism, providing contact between neighbouring cells,

between a cell and the ECM, or building up the paracellular barrier of epithelia to control the paracellular transport. In vertebrates, there are three major types of cell junctions — i.e., adherens junctions and desmosomes (Anchoring Junctions), gap junctions (Communicating Junctions), and tight junctions (Occluding Junctions). An adherens junction is defined as a cell junction whose cytoplasmic face is linked to the actin cytoskeleton, composed of cadherins and catenins. They can appear as bands encircling the cell (zonula adherens) or as spots of attachment to the ECM (adhesion plaques). Desmosomes attach the cell surface adhesion proteins to intracellular keratin cytoskeletal filaments, composed of desmoglein, desmocollin, desmoplakin, plakoglobin and plakophillin. Desmosomes help to resist shearing forces and are found in simple and stratified squamous epithelium and in muscle tissue as well. A gap junction directly connects the cytoplasm of two cells, which allows various molecules and ions to pass freely between cells. The gap junction hemichannels are primarily homo or hetero-hexamers of connecin proteins. Tight junctions are the closely associated areas of two cells whose membranes join together forming a branching network of sealing strands, composed of claudins and occludins to anchor the strands to the actin cytoskeleton. They help to maintain the polarity of cells and prevent the passage of molecules and ions through the space between cells [26].

Notably, less unique phosphopeptides were found from several cell junction proteins in CFPAC-1 cells (Tables 2 and 1S). Among these proteins, catenin beta 1, catenin delta 1 isoform 1ABC, catenin delta 1 isoform 1A, junction plakoglobin (gamma-catenin), and pleckstrin homology domain containing, family A member 7 are involved in adherens junctions [26,33]; plakophilin 2 isoform 2b, plakophilin 3, plakophilin 4 isoform a, desmoplakin isoform I are components of desmosomes; claudin 3, occludin, tight junction protein 3, and cingulin are associated with tight junctions [26,34]. Interestingly, in addition to their well-known roles, catenins have recently emerged as molecular sensors that integrate cell-cell junctions and cytoskeletal dynamics with signaling pathways that govern morphogenesis, tissue homeostasis, and intercellular communication [35].

Phosphorylation of cell structural proteins

The cytoskeleton provides the cell with structure and shape, and interacts extensively and intimately with cellular membranes. Eukaryotic cells contain three main kinds of cytoskeletal filaments, which are microfilaments, intermediate filaments, and microtubules. Microfilaments are the thinnest fibers of the cytoskeleton, approximately 6 nm in diameter. Each microfilament is made up of two helix, interlaced strands of actin subunits, and acts as a track for myosin motor motility. Many signal transduction systems use the actin cytoskeleton as a scaffold, holding them at or near the inner face of the peripheral membrane. Intermediate filaments, around 10 nanometers in diameter, organize the internal tridimensional structure of the cell, anchoring organelles and serving as structural components of the nuclear lamin and sarcomeres. They also participate in some cell-cell and cell-matrix junctions. Intermediate filaments are heterogeneous constituents of the cytoskeleton, made of vimentins, keratins, neurofilaments, and lamins. Microtubules are hollow cylinders about 23 nm in diameter, most commonly comprising 13 protofilaments which, in turn, are polymers of alpha and beta tubulin. They are commonly organized by the centrosome, and play key roles in the mitotic spindle and intracellular transport with the associated dyneins and kinesins [26]. In vivo microtubule dynamics vary considerably. Assembly, disassembly and catastrophe rate depend on which microtubule-associated proteins (MAPs) are present. MAP-microtubule binding is regulated through MAP phosphorylation by microtubule-affinity-regulating-kinase (MARK) [36].

As shown in Tables 2 and 1S, differential phosphorylation of cytoskeleton proteins and their associated proteins was revealed. First, more unique phosphopeptides were identified from several microfilaments-interacting proteins such as myristoylated alanine-rich protein kinase C substrate (MARCKS), drebrin 1 isoform a, phosphatase and actin regulator 4 isoform 1, whereas less unique phosphopeptides were identified from CDC42 effector protein 1 in CFPAC-1 cells. MARCKS is filamentous actin cross-linking protein, and it is the most prominent cellular substrate for protein kinase C. The protein is thought to be involved in cell motility, phagocytosis, membrane trafficking and mitogenesis [37]. Drebrin 1 is a cytoplasmic actin-binding protein thought to play a role in the process of neuronal growth [38,39]. CDC42 effector protein 1 has also been reported to be involved in the organization of the actin cytoskeleton [40]. Second, more unique phosphopeptides were identified from intermediate filament proteins such as nestin and nuclear lamin A/C isoform 1, whereas less unique phosphopeptides were identified from keratin 8, 18, 19, and 80 in CFPAC-1 cells. Third, 33 unique phosphopeptides were identified from microtubule-associated protein 1B (MAP-1B) in CFPAC-1 cells, but none were found in normal duct cells, indicating that either MAP-1B was not phosphorylated or the phosphorylation level was too low to be detected in normal duct cells in this study. In contrast, 5 unique phosphopeptides were identified from microtubule-associated protein 7 (MAP-7) in normal duct cells, but none were found in CFPAC-1 cells. Notably, some phosphorylation sites contain S/T-P motifs, indicating that mitogen-activated protein kinase (MAPK) was likely involved in phosphorylation of MAPs [41]. MAP-7, also named Ensconsin, play an important role in reorganization of microtubules during polarization and differentiation of epithelial cells [42,43]. MAP-1B is one of the major growth associated and cytoskeletal proteins in neuronal and glial cells. It is essential to stabilize microtubules during the elongation of dendrites and neuritis, and it can also interact with other cellular components, including filamentous actin and signaling proteins [44,45]. The functions of MAP-7 and MAP-1B are modulated by phosphorylation, and multiple phosphorylation sites have been previously identified in human cervix epithelial adenocarcinoma (Hela) cells by MS-based phosphoproteomic study [46–49]. In addition, differential phosphorylation was observed in stathmin 1 isoform a and transforming acidic coiled-coil containing protein 2 isoform d (TACC2). Stathmin 1 has been characterized as an important regulatory protein of microtubule dynamics. It interacts with two molecules of dimeric α, β-tubulin to form a tight ternary complex call the T2S complex. When stathmin sequesters tubulin into the T2S complex, tubulin becomes non-polymerizable [50,51]. Protein TACC2 has been implicated to play a role in organizing centrosomal microtubules [52]. Lastly, our MS result revealed differential phosphorylation of several motor proteins and nuclear pore proteins such as nucleoporin 88 kDa, nucleoporin 153 kDa, and kinesin light chain 3.

The reversible phosphorylation of proteins regulates almost all aspects of cell life since it underpins the process of signal transduction, and abnormal phosphorylation is a cause or consequence of many diseases such as cancer. In the past decade, researchers successfully profiled the phosphoproteomes of various mammalian cells and tissues and were able to identify thousands of phosphopeptides from over a thousand phosphoproteins by MS [46–49]. However, most of the reports have been focused on analysis of a single sample, and

information of comparative phosphoproteomic studies of cancer and normal samples was limited. Here, we exploited TiO_2-enrichment coupled to LC-MS/MS to identify the phosphoproteomes of PDAC cells and normal pancreatic duct cells, and qualitatively revealed differential phosphorylation from cell adhesion, cell junction and structural proteins. An understanding of composition and posttranslational modification of these proteins will certainly help in comprehending the complex dynamics of tumor invasion and metastasis in cancer biology. Furthermore, the identified phosphorylation sites can be used to map the signaling pathways that are involved in the regulation of these proteins and are potential targets for therapeutic development.

PDAC is characterized by a strong stromal presence, with 30–90% of tumor cells surrounded and interspersed by the fibroblastic stroma. As a result, some researchers used laser capture microdissection (LCM) to isolate enriched populations of cancer cells from the heterogeneous tissue specimen [3,11]. However, the phosphoproteomic analysis is limited by the relatively low number of cells that can be obtained from the capture for MS analysis and comparison. On the other hand, cell lines of PDAC are easily obtained, and the cultured cells are homogenous. In this study, we analyzed and compared a metastatic PDAC cell line with enriched normal duct cells obtained from primary culture of a tissue specimen. The cell line was used because of low yield of duct cells from primary culture of PDAC tissue. Although the proteins from cell lines are readily accessible and abundant enough for MS experiment, we need to keep in mind that data obtained with cell lines may not be representative of primary samples since the cell culture condition does not always reflect the tumor micro-environment. Thus, the preliminary findings from this comparison, as usual, require further investigations to determine their relevant roles in the PDAC cells *in vivo*, and validation of the differences observed. While our analysis was comprehensive, another limitation of our study is the potential for biological variability–since only 2 samples were compared, the extension of these results to more broad-based hypothesis is not possible. This is worthwhile for future work to analyze and compare different cell lines and normal duct cells from more human subjects in order to understand the significance of the findings to date.

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