Mass spectrometry-based iTRAQ analysis of serum markers in patients with pancreatic cancer

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Abstract. Early biomarkers for pancreatic cancer (PC) detection are required in order to improve patient outcomes. The present study aimed to identify serum biomarkers for PC diagnosis using proteomics, unveil the underlying pathological mechanisms and provide reliable data for the early diagnosis of PC. Isobaric tags for relative and absolute quantification and two-dimensional-liquid chromatography-tandem mass spectometry were used to compare serum samples from patients with PC and healthy individuals. Mascot and Scaffold were used for raw data processing, and Panther for gene ontology (GO) analysis. Igenuity® Pathway Analysis (IPA) was utilized to assess canonical pathways and protein-protein interactions. In total, 76 differentially expressed proteins were identified. The candidate protein DNA repair protein 50 (RAD50) was elevated in patients with PC compared with healthy individuals. In addition, transforming growth factor-β1 (TGF-β1) and apoptotic protease activating factor 1 (APAF-1) were downregulated in PC. GO analysis revealed that the extracellular matrix was increased in PC, as well as receptor function and enzyme regulation; additionally, reduced nucleic acid binding transcription factor activity was observed. IPA analysis demonstrated that the significantly altered canonic pathways were liver X receptor/retinoid X receptor (RXR) activation, the production of nitric oxide and reactive oxygen species in macrophages, the coagulation system, acute phase response signaling and lipopolysaccharide/interleukin-1 mediated inhibition of RXR function. To conclude, RAD50, TGF-β1 and APAF1 are candidate biomarkers for the diagnosis of early PC. The results from the present study could help identify future therapeutic drugs for PC.

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Abbreviations: PC, pancreatic cancer; iTRAQ, isobaric tags for relative and absolute quantification; GO, gene ontology

Key words: pancreatic cancer, biomarker, isobaric tags for relative and absolute quantification analysis, mass spectrometry, serum

Introduction

Pancreatic cancer (PC) is an aggressive malignant tumor with a low survival rate; the 5-year survival rate of this malignancy is <8%, making it the third major cause of cancer-associated mortality in the USA (1). It has been predicted that PC will become the second leading cause of cancer-associated mortality after lung carcinoma by 2030 (2). Such high mortality is caused by delayed diagnosis due to a lack of early diagnostic tools. Indeed, the early diagnosis of PC serves a vital role in disease prognosis and the design of personalized therapy. Due to the lack of overt symptoms at the early stage of PC, missed diagnosis rates range between 39 and 70% (3). Patients with advanced stages of PC demonstrate a more severe physical condition compared with those diagnosed in the early stage (4). In addition, certain tumor biomarkers play important roles in the early diagnosis, therapy and monitoring of PC, such as carbohydrate antigen (CA)19-9, CA242, vascular endothelial growth factor (VEGF), CA724 and CA125. However, although the levels of the aforementioned tumor biomarkers are elevated in certain individuals with PC, this is not the case for all, and so they have low levels of sensitivity and specificity (5). More strikingly, CA19-9 is a biomarker approved by the US Food and Drug Administration for PC diagnosis, with a sensitivity of 70-81%; however, it fails to detect PC in the early stages of disease (6). Therefore, new tumor biomarkers that serve significant roles in the early diagnosis of PC are urgently needed.

Currently, proteomics is considered a powerful tool for accurate monitoring and quantification of protein expression changes (7). Indeed, several proteomic technologies have been widely used for the identification of biomarkers, including two-dimensional electrophoresis, stable isotope labeling with amino acid in cell culture, two-dimension difference gel electrophoresis and isobaric tags for relative and absolute quantification (iTRAQ) (8). Previously, iTRAQ-based analysis has been used to quantitatively assess the changes in protein abundance in various biological samples, with high sensitivity and reproducibility (9). For instance, this approach has been successfully used to identify diagnostic markers of gastric and lung cancer (8,9).

The iTRAQ technology is increasingly used in the field of cancer research; plasma or serum protein analysis via quantitative proteomics of patients with PC reveals cancer-associated proteins and polypeptides in comparison with specimens from non-diseased and chronic pancreatitis controls (10). Thus, the present study aims to assess serum proteins in patients with PC and healthy controls, providing a basis for screening serum biomarkers, which would be used in the early diagnosis of PC.

Materials and methods

Subjects. A total of 15 freshly collected serum specimens were obtained from patients (mean age, 51.70±3.10 years; all male) diagnosed with PC (without hypertension or diabetes) between January 2013 and January 2014 at Shanxi Provincial Cancer Hospital (Taiyuan, China). The samples were obtained prior to treatment (Fig. 1). The diagnosis criteria were based on the Guidelines for Diagnosis and Treatment of Pancreatic Cancer (2014 Edition) from Group of Pancreatic Surgery, Branch of Surgery, Chinese Medical Association (11). The American Joint Committee on Cancer (AJCC) Tumor-Node-Metastasis (TNM) staging is the most widely used cancer staging system worldwide. According to the AJCC 8th edition staging system for patients with PC, 7 patients had been diagnosed as stage IIB, and 8 patients had been diagnosed as stage III (12) (Table I). The tumor status of 15 patients with PC are shown in Table II. During the same period, 10 healthy control subjects undergoing physical examinations at Shanxi Provincial Cancer Hospital (mean age, 56.40±2.42 years; all male) were enrolled. The present study was approved by The Ethics Committee of Shanxi Provincial Cancer Hospital (approval no. 201732), and written informed consent was obtained from each participant.

Serum collection and preservation. Blood samples were collected in the morning before the patients had eaten, placed at 4˚C for 1.5 h, and centrifuged at 3,000 x g for 10 min at 4˚C. The resulting supernatant (serum) was collected and stored at -80˚C.

Depletion of highly and lowly abundant proteins. The serum samples from 15 patients and 10 healthy controls collected at baseline were immuno-affinity depleted of highly abundant proteins using a Multiple Affinity Removal Column Human 14 (4.6x50 mm; Agilent), according to the manufacturer's protocol. Lowly abundant proteins were collected on a high-performance liquid chromatography (HPLC) system (1260 Infinity II LC System; Agilent) using dilution, stripping and neutralization buffers provided, according to the manufacturer's protocol. The detailed conditions were: Column, Agilent HC-C18 (4.6x150 mm, 5 μm); mobile phase, methanol/water 20:80 (v/v) containing 0.05 M potassium dihydrogen phosphate (pH 3.5); detection wavelength, 238 nm; flow rate, 1.0 ml/min; column temperature, 35˚C; injection volume, 20 μl. To 200 μl plasma, 600 μl 10% perchloric acid was added; after mixing, the sample was centrifuged at 16,000 x g for 10 min on a GL-20G-II high-speed desktop refrigerated centrifuge (Flying Pigeon; Shanghai Anting Scientific Instrument Factory, Shanghai). Following this, 20 μl of the resulting supernatant was used for quantitative analysis.

Protein quantification and SDS-PAGE electrophoresis. The serum original samples of patients with pancreatic cancer and healthy controls were analyzed by SDS-PAGE. Serum protein levels were determined using a Bradford assay kit (Beyotime Institute of Biotechnology). The depletion efficiency of each approach was evaluated by SDS-PAGE. A Coomassie Blue Stain kit PH0351 (Feijing Scientific Research Reagent Store) was used containing 100 ml Coomassie Blue stain and 500 ml Coomassie Blue decoloring solution. Coomassie blue staining was performed at 23˚C for 2-4 h followed by decolorizing at 23˚C for 4-8 h. The type of gel was SDS-PAGE gels. No two-dimensional separation was used. Then, equal amounts (15 μg) of total protein were separated by 12% SDS-PAGE electrophoresis.

Peptide extraction and iTRAQ labeling. In total, 200 μg of highly abundant protein depleted serum was denatured with 200 μl dithiothreitol (20 mmol/l) at 37˚C for 1 h, and alkylated with iodoacetamide (50 mmol/l) at room temperature for 45 min. Urea (8 mol/l) was used for elution, and ammonium bicarbonate (25 mmol/l) for washing. Finally, trypsin was used overnight to digest the serum proteins at 37˚C, and the reactions were terminated by addition of formic acid at a final concentration of 1%. Next, iTRAQ labeling was performed for peptides from healthy controls and PC samples using an iTRAQ reagent 8-plex kit (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Peptides were desalted, dried and labeled with different isobaric tags for 2 h at 25˚C. Peptides enriched in serum samples from healthy controls and patients with PC were labeled with 116 and 117 tags, respectively. Finally, the labeled peptides were dried under vacuum and stored at -80˚C.

Separation of iTRAQ-labeled peptides under high pH reversed phase (RP) conditions. Digestion and iTRAQ labeled serum samples were reconstituted in 400 μl of 20 mm ammonium formate/2% acetonitrile (pH 10). Following this, the samples were loaded on an RP C18 capillary column (3, 4.6 μmx250 mm; Shimadzu Corporation) and fractionated on an Agilent 1100 series HPLC instrument by basic RP chromatography at a flow rate of 1.0 ml/min. The mobile phase consisted of 90% acetonitrile (pH 10), used with a gradient of 5 to 30% solvent B (formic acid), for 0-60 min. Fractions were collected every 1 min for a total of 60 fractions; early and late fractions were pooled, resulting in a total of 30 fractions. The pooled samples were reconstituted in 0.1% formic acid for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

LC-MS/MS analysis. Peptides in each fraction were separated on an RP C18 capillary column (3, 75 μmx100 mm) and eluted using a linear gradient of 5 to 30% solvent B1 (0.1% v/v) formic acid in acetonitrile for 40 min, at a flow rate of 300 nl/min). MS data were acquired by the shotgun proteomics method (LTQ-Orbitrap mass spectrometer; Sanofi S.A.); in each cycle, a full scan was acquired at a resolution of 30,000 dpl with a mass range of 380-1,600 m/z. Up to 10 of the most intense precursor ions with charge range of +2 to +4 were selected with an isolation window of 2 Da, and subsequently fragmented by higher-energy collisional dissociation with a normalized

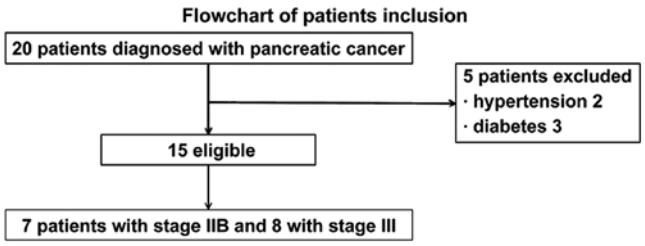

Figure 1. Flow chart presenting the inclusion criteria for patients within the present study.

collision energy of 40%. The MS/MS scans were acquired at a resolution of 7,500 dpl. Precursor ions were dynamically excluded from reselection for 60 sec.

Database analysis. Raw data from the mass spectrometer were analyzed by the Mascot (version 2.3.02; http://www.matrixscience.com/) and Scaffold (version 4.4.3; Proteome Software, Inc.) programs. The search database was Swiss-Prot (uniprot.org) (human species). Tyrosine served as a variable modifier protein. The search parameters were: Trypsin allowed P enzyme specificity, with up to two missing cleavages; precursor ion mass tolerance, ±10 ppm; fragment ion mass tolerance, 0.05 Da; fixed modification, carbamidomethylation and no variable modification. The identified proteins were quantified according to the iTRAQ of the specific polypeptide. Differential proteins in serum proteome: Differential multiple >1.5, P<0.05.

Gene ontology (GO) and Igenuity® Pathway Analysis (IPA) analyses of differentially expressed proteins. Differentially expressed proteins (ratio >1.5 or <0.5; P<0.05) were imported into the Panther database (pantherdb.org/) and matched with the human genome data. Following this, the proteins were classified into molecular function, biological process and cellular component according to gene ontology (GO). Canonical pathway, upstream regulation and protein-protein interaction analyses were performed by the IPA software (Ingenuity Systems; Qiagen, Inc.).

Statistical analysis. Data are presented as mean ± standard deviation. SPSS 19.0 software (SPSS; IBM Corp.) was used to analyze the results. Student's t-test was applied to compare mean values between the two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Characteristics of patients with PC. As presented in Table III, serum CA19-9, CA242, CA724, CA50 and VEGF levels in patients with PC were significantly increased compared with the values of healthy controls. In addition, these diagnostic factors were higher than normal levels, except VEGF.

Qualitative and quantitative analyses of serum proteins following immuno-affinity depletion of highly abundant proteins. Serum samples from patients with PC and control patients were immuno-affinity depleted of the 14 most abundant proteins using Multiple Affinity Removal Column Human 14, and SDS-PAGE was performed to assess various serum samples in the two patient groups. As presented in Fig. 2, low abundance proteins demonstrated improved separation and exhibited higher concentrations. In addition, an increased number of low abundance proteins was observed; however, the albumin band disappeared. In the serum proteome, 251,479 spectra were obtained. Proteomics revealed 531 proteins, of which 442 were quantifiable.

Table I. Number of patients with pancreatic cancer at each stage of the 8th edition of the American Joint Committee on Cancer staging system (12) for pancreatic cancer.

Variables	Number of patients
Tumor depth	
T1	4
T2	7
T3	4
Lymph node metastasis	
N1	7
N2	8
Distant metastasis	
M0	15
Tumor stage	
IIB	7
III	8

N, node; M, metastasis.

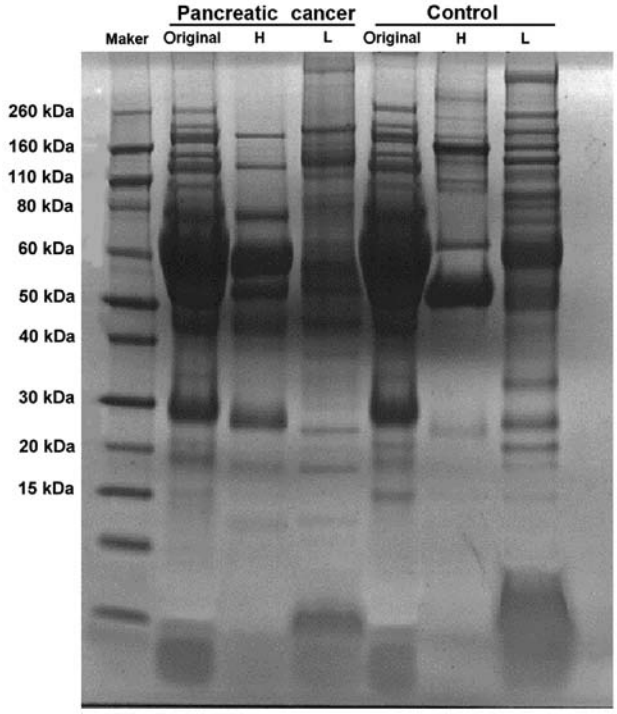

Figure 2. SDS-PAGE analysis of the serum original sample of patients with pancreatic cancer and healthy controls. Original, original serum; L, no high abundance proteins in serum; H, high abundance proteins in serum.

Table II. Tumor status of 15 patients with pancreatic cancer included in the present study.

Patient no.	Sex	Tumor size, cm	Tumor site	Degree of differentiation	Lymph node Metastasis numbers	T	N	M	Stages
1	Male	3x2.5x2.5	Head of pancreas	Moderately	3	2	1	0	IIB
2	Male	1.8x1.8	Tail of pancreas	Moderately	1	1	1	0	IIB
3	Male	3x2x0.5	Tail of pancreas	Moderately/poorly	2	2	1	0	IIB
4	Male	1.8x1.8	Head of pancreas	Moderately	2	1	1	0	IIB
5	Male	2.5x2.5x2	Head of pancreas	Moderately	3	2	1	0	IIB
6	Male	1.5x1.5	Head of pancreas	Moderately	1	1	1	0	IIB
7	Male	2x2x1.5	Body of pancreas	Moderately	2	1	1	0	IIB
8	Male	3x3x3	Body of pancreas	Highly/moderately	5	2	2	0	III
9	Male	3x3x3	Head of pancreas	Moderately	4	2	2	0	III
10	Male	3x2.5x2.5	Head of pancreas	Moderately/poorly	6	2	2	0	III
11	Male	5x3.7x3.5	Head of pancreas	Moderately	8	3	2	0	III
12	Male	4x3x3	Head of pancreas	Moderately	4	2	2	0	III
13	Male	4x3.5x3.5	Body of pancreas	Moderately	5	2	2	0	III
14	Male	5x3x3	Head of pancreas	Poorly	9	3	2	0	III
15	Male	5x4x4	Head of pancreas	Highly	8	3	2	0	III

T, Tumor; N, Node; M, Metastasis.

Table III. Clinical data and carbohydrate antigen levels in healthy controls (male) and patients with pancreatic cancer (male) (mean ± SD)

Variable	Healthy control group (n=10)	Patients with pancreatic cancer group (n=15)	Reference values	P-value
Age, years	51.70±3.10	56.40±2.42	-	-
Complications	None	None	-	-
CA19-9	5.05±2.01 U/ml	251.3±63.87 U/ml	<20 U/ml	P<0.01
CA242	2.12±0.71 U/ml	28.76±13.82 U/ml	<12 U/ml	P<0.01
CA724	4.31±1.29 U/ml	12.76±7.62 U/ml	<10 U/ml	P<0.05
CA50	5.48±1.46 U/ml	176.0±60.71 U/ml	<20 U/ml	P<0.05
VEGF	185.0±60.36 pg/ml	365.0±39.67 pg/ml	62-707 pg/ml	P<0.05
Total, n	10	15	-	-
Age, years	51.70±3.10	56.40±2.42	-	-
Complications	None	None	-	-

CA, carbohydrate protein; VEGF, vascular endothelial growth factor.

GO analysis. Based on the aforementioned 442 quantifiable proteins, more than two peptide segments were identified by Mascot and Scaffold. There were 76 differentially expressed proteins in samples from patients with PC compared with control specimens (Table IV). Among them, 70 proteins were downregulated, with 24 demonstrating a coordination ratio of 0.4 or more. Meanwhile, six proteins were upregulated by 2-3 fold. To comprehensively assess the biological significance of the 76 differentially expressed proteins, analysis was performed by the Panther gene classification system, which includes biology processes, cellular components, molecular functions and protein classes. Compared with the normal serum proteome database (pantherdb.org/chart/summary/pantherChart.jsp?filterLevel=1&chartType=1&listType=1&type=5&species=Homo%20sapiens), proteins associated with the extracellular matrix, receptor activity and enzyme regulator activity were upregulated in patients with PC, and those involved in the extracellular matrix accounted for 6.8% of all proteins (Fig. 3). However, proteins involved in nucleic acid binding transcription factor activity and metabolic processes were significantly downregulated.

Pathways identified by IPA. A bioinformatics analysis of the identified differentially expressed proteins was performed using the IPA software. As presented in Fig. 4, certain pathways were significantly altered. Proteins in the coagulation system (P=4.97; ratio, $1.43x10^{-1}$) and lipopolysaccharide/interleukin-1

Table IV. Analysis of the 76 differentially expressed proteins identified in the serum of patients with pancreatic cancer and healthy controls.

A, Upregulated proteins

Protein	ID (Swiss-Prot)	Change fold
Cluster of transmembrane protease serine 13	Q9BYE2	3
Triose-phosphate isomerase	P60174	2.5555556
Complement factor H-related protein 2	P36980	2.5
Lysozyme C	P61626	2.2222222
DNA repair protein RAD50	Q92878	2.2
WD repeat-containing protein 67	Q96DN5	2

B, Downregulated proteins

Protein	ID (Swiss-Prot)	Change fold
Cadherin-related family member 2	Q9BYE9	0.4545455
Apolipoprotein C-I	P02654	0.4545455
Cluster of membrane primary amine oxidase	Q16853	0.4545455
A-kinase anchor protein 13	Q12802	0.4444444
Cluster of Ig κ chain V-III region WOL	P01623	0.4166667
Titin	Q8WZ42	0.4
Ig κ chain V-I region EU	P01598	0.4
Polymeric immunoglobulin receptor	P01833	0.4
Mast/stem cell growth factor receptor kit	P10721	0.4
Collagen α-1(I) chain	P02452	0.4
Roundabout homolog 4	Q8WZ75	0.4
G-protein coupled receptor 126	Q86SQ4	0.4
Protein RRP5 homolog	Q14690	0.4
Plasminogen activator inhibitor 1	P05121	0.4
4F2 cell-surface antigen heavy chain	P08195	0.4
Plexin-B1	O43157	0.4
Sushi, nidogen and EGF-like domain-containing protein 1	Q8TER0	0.4
Multiple epidermal growth factor-like domains protein 8	Q7Z7M0	0.4
Androgen receptor	P10275	0.4
TRAF3-interacting protein 1	Q8TDR0	0.4
NACHT, LRR and PYD domains-containing protein 6	P59044	0.4
Ankyrin repeat domain-containing protein 30A	Q9BXX3	0.4
Collagen α-1(XVI) chain	Q07092	0.4
Protein QN1 homolog	Q5TB80	0.4
α-amylase 1	P04745	0.375
Ig heavy chain V-III region GAL	P01781	0.3636364
A disintegrin and metalloproteinase with thrombospondin motifs 13	Q76LX8	0.3636364
Proteasome subunit β type-4	P28070	0.3636364
Endoglin	P17813	0.3636364
Leukocyte elastase inhibitor	P30740	0.3636364
Sodium channel protein type 8 subunit α	Q9UQD0	0.3636364
Desmoglein-2	Q14126	0.3333333
Msx2-interacting protein	Q96T58	0.3
Unconventional myosin-XV	Q9UKN7	0.3
Serum amyloid A-4 protein	P35542	0.3
Apolipoprotein C-IV	P55056	0.3
Golgi-associated plant pathogenesis-related protein 1	Q9H4G4	0.3
Vascular endothelial growth factor receptor 3	P35916	0.3
Kinesin-like protein KIF20B	Q96Q89	0.3

Table IV. Continued.

B, Downregulated proteins

Protein	ID (Swiss-Prot)	Change fold
Centromere protein F	P49454	0.3
Ig heavy chain V-III region JON	P01780	0.3
Leukemia inhibitory factor receptor	P42702	0.3
Cingulin-like protein 1	Q0VF96	0.3
G protein-coupled receptor kinase 5	P34947	0.3
Plasma serine protease inhibitor	P05154	0.2727273
Plexin-D1	Q9Y4D7	0.2727273
Lactotransferrin	P02788	0.25
Pericentrin	O95613	0.25
Dystonin	Q03001	0.25
Serine-protein kinase ATM	Q13315	0.2307692
Pyruvate dehydrogenase (lipoamide) kinase isozyme 2, mitochondrial	Q15119	0.2307692
Serine/arginine repetitive matrix protein 1	Q8IYB3	0.2222222
Apolipoprotein C-III	P02656	0.2
Hornerin	Q86YZ3	0.2
Protein shroom2	Q13796	0.2
Transmembrane protein 74	Q96NL1	0.2
Serine/threonine-protein kinase WNK4	Q96J92	0.2
RING finger protein 214	Q8ND24	0.1818182
Midasin	Q9NU22	0.1666667
Dynein heavy chain 11, axonemal	Q96DT5	0.1666667
Regulatory-associated protein of mTOR	Q8N122	0.1538462
Zinc finger ZZ-type and EF-hand domain-containing protein 1	O43149	0.1538462
Poly (ADP-ribose) polymerase 1	P09874	0.125
Probable ATP-dependent RNA helicase DDX46	Q7L014	0.125
Peregrin	P55201	0.125
Semaphorin-4B	Q9NPR2	0.117647
Nesprin-1	Q8NF91	0.1
Canalicular multispecific organic anion transporter 1	Q92887	0.1
Apoptotic protease-activating factor 1	O14727	0.1
Sodium/hydrogen exchanger 2	Q9UBY0	0.1

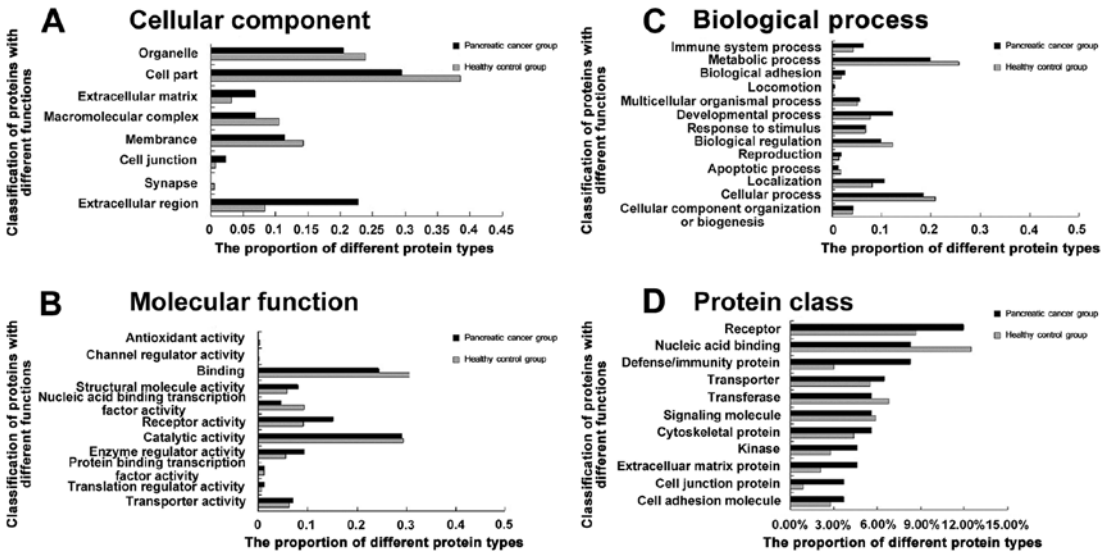

Figure 3. Gene Ontology analysis of differentially expressed proteins in immuno-affinity depleted serum. (A) Cellular components. (B) Molecular functions. (C) Biological process. (D) Protein class. The y-axis displays different functions, while the x-axis presents the proportion of different protein types. Black, pancreatic cancer group; gray, healthy control group.

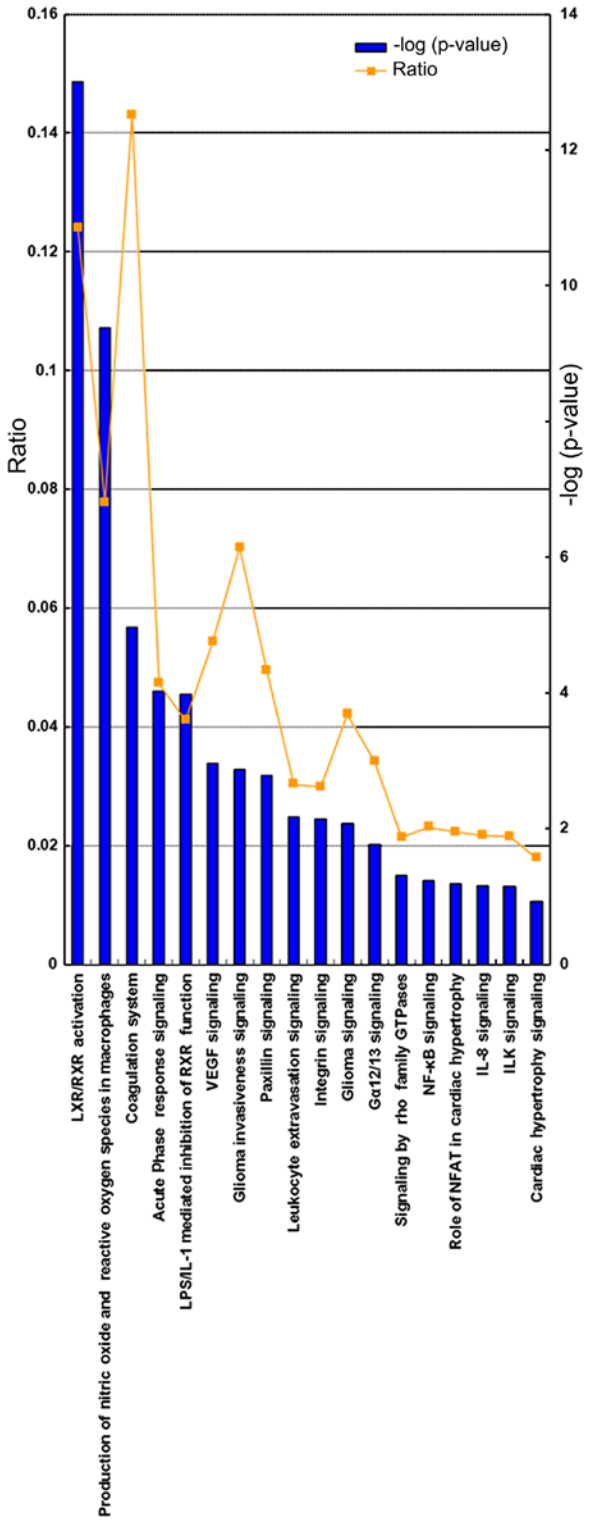

Figure 4. Analysis of biological pathways of the differentially expressed proteins in patients with pancreatic cancer and healthy controls using the Ingenuity Pathway Analysis software. -Log(P-value) reflects the significance between proteins and the pathway. The higher the score, the higher the degree of significance. LXR, liver X receptor; RXR, retinoid X receptor; LPS, lipopolysaccharide; IL, interleukin; VEGF, vascular endothelial growth factor; NF-κB, nuclear factor κB; NFAT, nuclear factor of activated T-cells; ILK, integrin linked kinase.

mediated inhibition of retinoid X receptor (RXR) function (P=3.98; ratio, $4.11x10^{-2}$) were significantly upregulated. In comparison, liver X receptor/RXR activation (P=13.00; ratio, $1.24x10^{-1}$), production of nitric oxide and reactive oxygen species in macrophages (P=9.38; ratio, $7.78x10^{-2}$), paxillin signaling (P=2.78; ratio, $4.95x10^{-2}$), and integrin signaling (P=2.14; ratio, $2.99x10^{-2}$) were significantly downregulated. In addition, some differentially expressed proteins were involved in cell survival, molecular translocation, viral infection and lipid efflux, revealing decreased levels. A protein interaction network analysis also revealed that certain associated proteins were upregulated or downregulated (Fig. 5).

Discussion

CA199 is a tumor-associated antigen (13). It is present in the majority of healthy individuals. Not all individuals are able to produce similar levels of CA19-9; the levels are closely associated with physiological characteristics and genetic status (13). The physiological level of CA19-9 varies from individual to individual due to their different genotypes. In the serum of those with Lewis (Le) antigen Le-a (+) and Le-b (-) genotypes, CA19-9 is maintained at a certain level (14). The serum CA19-9 level of Le-a (-), Le-b (+) genotype was relatively low. CA19-9 could not be detected in the serum of individuals with Le-a (-), Le-b (-) genotype. In individuals who are negative for Lewis antigen, CA19-9 was not significantly elevated despite the PC tumor being large. Despite CA19-9 being a PC-specific antigen, its specificity and sensitivity to the PC tumor are low, which makes it difficult to use CA19-9 as a biomarker in screening for the early diagnosis of PC, although it is a common method (14). CA19-9 has been detected in various other types of cancer, including pancreatic (84%), gallbladder (69%), colorectal (39%) and ovarian (35%) cancer (15). Pancreatitis, hepatitis, biliary inflammation and obstructive diseases have been reported as the most common benign diseases exhibiting elevated levels of CA19-9 (15). The diagnostic sensitivity of CA19-9 for PC decreased when the critical value of CA19-9 increased and when the specificity level increased. When the critical value of CA19-9 reached 100 ku/l, the sensitivity and specificity were 68 and 98%, respectively (14). Even though CA19-9 is considered a good diagnostic marker for PC, it should not be used for screening of the early stages of PC, as the levels of CA19-9 are rarely elevated in this stage (14). Even when the PC lesions have been detected using CT, but the tumor size was <3 cm, only 50% of the patients had elevated CA19-9 (14). The most important role that CA19-9 serves in PC is to evaluate the recurrence of PC and the efficacy of radiotherapy and chemotherapy following surgery via assessing the changes in the levels of CA19-9. Despite the level of CA19-9 in patients with PC being increased >10 times in the present study, it could not be used as a direct indicator for the diagnosis of PC.

The present study assessed serum proteins in patients with PC and healthy controls and identified a total of 531 proteins using iTRAQ technology. Among them, 442 were quantitatively analyzed. Several differentially expressed proteins were observed in subjects with PC in comparison with healthy controls, including APAF-1, RAD50 and TGF-β1. GO and IPA analyses demonstrated that the differentially expressed proteins mainly participated in the extracellular matrix component, receptor function, enzyme regulation, nucleic acid binding transcription factor activity and metabolic-associated

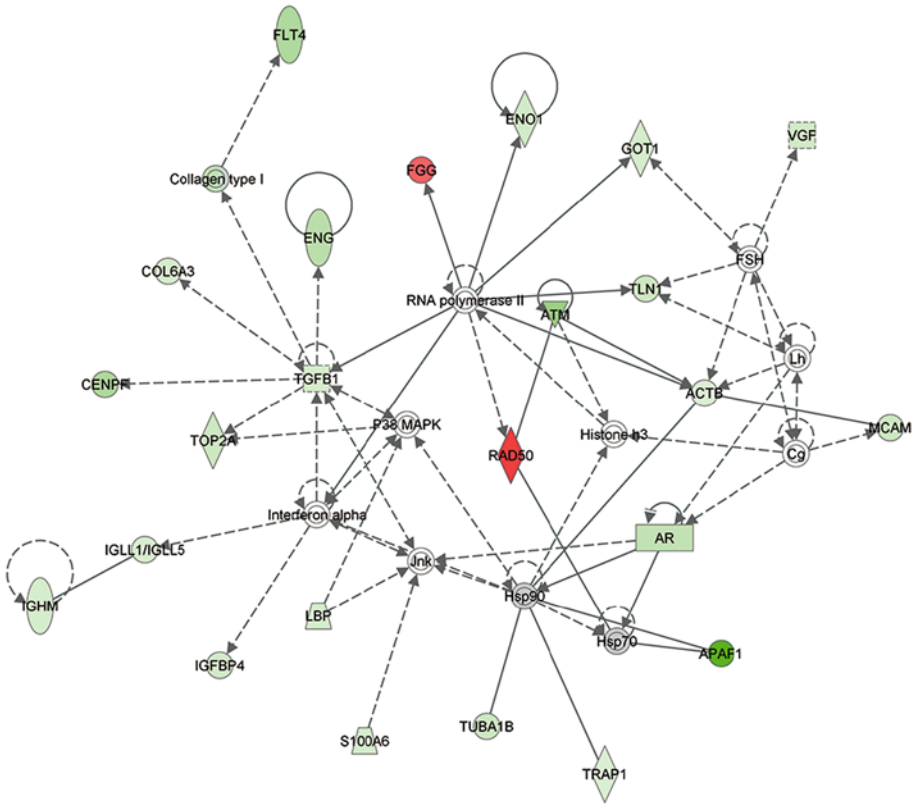

Figure 5. Interaction network of differentially expressed proteins between patients with pancreatic cancer and healthy controls, assessed by the Ingenuity Pathway Analysis software. Red shapes indicate upregulated proteins, and green shapes indicate downregulated proteins.

biological process. In addition, protein-protein interaction analysis using the IPA software also revealed marked alterations of APAF-1, RAD50 and TGF-β1 in patients with PC.

As presented in Fig. 5 and Table IV, APAF-1 was significantly downregulated by 10-fold in serum samples from patients with PC compared with the normal group. It has previously been demonstrated that APAF-1 is an essential and necessary component of apoptotic bodies in the intrinsic apoptotic pathway (16). APAF-1 serves an important role in the mitochondrial apoptotic pathway, primarily affecting the normal apoptosis of cells (17). Loss of heterozygosity and DNA methylation can inactivate APAF-1, which is a common phenomenon in numerous types of cancer in humans, including PC (18,19). It has also been reported that when APAF-1 is inhibited in the pancreatic duct, it can result in the development of PC (17).

As an important component of the Mre11-Rad50-Nbs1 complex (20), RAD50 is primarily involved in maintaining chromosome stability. In the present study, RAD50 was increased by 2.2-fold in serum samples from patients with PC compared with the normal group. A mutation analysis of 32 double-stranded DNA damage repair genes in breast cancer and PC revealed that RAD50 inactivation was more likely to promote the development of PC (21); however, high expression of RAD50 is conserved (22). It is not yet understood whether RAD50 protein upregulation in patients with PC is a protected molecular mechanism or whether the upregulation is maintained via negative feedback regulation; further research is required in order for the underlying mechanism to be elucidated.

TGF-β plays an important role in cell cycle arrest, apoptosis, homeostasis, wound healing and immune regulation. In the case of cancer, TGF-β plays a dual role in different situations, functioning as a tumor suppressor or oncogene in the early disease stages (23). TGF-β has three isoforms, including TGF-β1, TGF-β2 and TGF-β3; among them, TGF-β1 is the most abundant in humans. The TGF-β signaling pathway consists of several stages, starting with TGF-β1 activation and release, followed by binding to three high affinity receptors (24). TGF-β binds to TGF-βR1 and TGF-βR2 receptors on the cell surface, forming dimeric-SMADs following activation. A previous study demonstrated that TGF-βR2 is one of the 16 most commonly mutated genes in PC (25). In multiple types of human cancer, the TGF-β signaling pathway is activated (26). As presented in Table IV, TGF-β1 was 1.67-fold less expressed in serum samples from patients with PC compared with control values. Through IPA analysis of upstream regulatory genes of TGF-β1, it was also revealed that significantly downregulated TGF-β1 may be due to the suppression of hypoxia inducible factor 1 (HIF1) α. According to a previous report, HIF1α plays important roles in the growth, invasion and metastasis of PC (27). Significant changes in TGF-β1 expression indicate that TGF-β1 may be one of the diagnostic indices for PC.

The present study demonstrated APAF-1, RAD50 and TGF-β1 should be considered candidate biomarkers for PC diagnosis. However, due to the limited sample size, further studies with larger sample sizes are required in order to confirm the identified differentially expressed proteins and validate their values in the diagnosis of PC. The results of the

present study could aid in identifying future therapeutic drugs for the treatment of PC.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

KJ and XD conceived and designed the study. KJ and XZ performed the experiments and data analyses. KJ wrote the manuscript. KJ and XD helped to revise the manuscript. XZ contributed to discussions and provided reagents/materials/analysis tools important for the completion of this work.

Ethics approval and consent to participate

All procedures performed in the present study that involved human participants were in accordance with the ethical standards of the institutional and/or national Ethics Committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The present study was approved by the ethics committee of Shanxi Provincial Cancer Hospital (approval no. 201732). All the patients included in the present study provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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