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ORIGINAL ARTICLE

Proteomic analysis of pancreatic intraepithelial neoplasia and pancreatic carcinoma in rat models

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Abstract

AIM: To detect the proteomic variabilities of pancreatic intraepithelial neoplasia (PanIN) and pancreatic carcinoma (PC) induced by 7,12-dimethylbenzanthracene (DMBA) in rat models and to identify potential biomarkers.

METHODS: Sixty adult male Sprague Dawley rats were randomized into three groups. The rats had DMBA implanted into their pancreas for one (n = 20) or two months (n = 20) or assigned to the normal group (n = 20). The rats were killed after one or two months, and were evaluated histopathologically. Three tissue samples from each group of rats with either normal pancreas, PanIN (PanIN-2) or PC were examined by 2D-DIGE. The different expression spot features were analyzed by matrix-assisted laser desorption/ionization-time of flight/time of flight (MALDI-TOF/TOF) tandem mass spectrometry. The expression of enolase 1, a differentially expressed protein, was identified by immunohistochemistry.

RESULTS: There was significant difference in the proportions of neoplastic changes between the 1- and 2-mo groups (P = 0.0488). There was an increase in the frequency of adenocarcinomas in the 2-mo group compared with the 1-mo group (P = 0.0309). No neoplastic changes were observed in any of the animals in the normal group. Enolase 1, pancreatic ELA3B, necdin, Hbp23, CHD3, hnRNP A2/B1, Rap80, and Gnb2l1 were up-regulated in the PanIN and PC tissues, and CEL, TPT1, NME2, PCK2, an unnamed protein product, and glycine C-acetyltransferase were down-regulated in the PanIN and PC tissues. The immunohistochemical results showed that enolase 1 expression was up-regulated in the pancreatic cancer tissues of rats and humans.

CONCLUSION: The pancreatic protein expression changes induced by DMBA suggest potential molecular targets for the early diagnosis and treatment of PC.

Key words: 7,12-dimethylbenzanthracene; Pancreatic intraepithelial neoplasia; Pancreatic carcinoma; Proteomics

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INTRODUCTION

Pancreatic carcinoma (PC) is one of the most lethal hu-

man cancers, and 80%-90% of PCs are pancreatic ductal adenocarcinomas. Pancreatic cancer is characterized by a late presentation, and for all stages of this cancer, the five-year survival rate is less than 5%. It is currently the fourth most common cause of cancer death for both men and women[1]. This poor prognosis relates to the uniformly advanced disease stage at the time of diagnosis and its profound resistance to the existing therapies. An early detection of pancreatic cancer is required to improve its poor prognosis.

As the clinical diagnosis of pancreatic cancer is mostly at advanced stages, to study the mechanisms of PC and screen tumors for early diagnostic markers, it is important to establish animal models of precancerous pancreatic lesions and the early stages of PC with pathological characteristics resembling that of human PC. Pancreatic intraepithelial neoplasia (PanIN) has been identified as the precursor of pancreatic ductal adenocarcinoma[2,3], and animal models of PanIN and pancreatic ductal adenocarcinoma can provide opportunities to investigate earlier pancreatic cancers that are rare in human samples. Animal models involve successful chemical carcinogens such as 7,12-dimethylbenzanthracene (DMBA). DMBA implantation into the pancreas is known to induce ductal PanIN and PC in mice and rats through pathways that consistently involve K-ras gene mutations and the activation of Notch signaling, as in human pancreatic cancer[4-10]. At present, DMBA-induced pancreatic carcinogenesis models have been used in the identification of possible promoters or suppressors of PC[8,11,12], stable isotope glucose-tracer studies[13] and molecular analyses[10].

Proteomic techniques are emerging as important tools for studying the mechanisms of disease and finding potential biomarkers and new therapeutic targets, which enable investigators to determine whether a particular protein level is increased or decreased when comparing two different conditions (e.g. a diseased state and a non-diseased state)[14,15]. Although proteomic studies of PC in human samples have been reported, most of the samples were acquired from advanced PC but not precancerous pancreatic lesions or the early stages of PC. The protein expression changes from normal pancreas to PanIN and early stages of PC remain unclear. In the present research, we studied the proteomic variabilities of rat models of DMBA-induced PanIN and PC using proteomic techniques, two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) and mass spectrometry, to reveal new potential biomarkers for PanIN and PC.

MATERIALS AND METHODS

Animal treatment

Sixty adult male Sprague Dawley (SD) rats (100-110 g) were randomized into three groups. The rats in the 1- (n = 20) and 2-mo groups (n = 20) had DMBA (10 mg/100 g) directly implanted into their pancreas, according to a previously established protocol[9], and the surviving rats were killed after one month and two months, respectively. A normal group of 20 rats was killed after two months. The carcinogen implant site was separated from the rest of the pancreas, the pancreatic nodules were fixed in formalin and embedded in paraffin, and multiple 5-mm sections were prepared and stained with hematoxylin and eosin for routine histological examinations. The pancreas slides were reviewed by a single pathologist and evaluated histologically according to the PanIN classification system[2,3].

Table 1 Sample markers and gel distribution in 2D-DIGE analysis

Gel	Cy2	Cy3	Cy5
Gel1	Internal standard	A1	B3
Gel2	Internal standard	B3	C2
Gel3	Internal standard	C1	A2
Gel4	Internal standard	B2	C3
Gel5	Internal standard	A3	B1

2D-DIGE and image analysis

Three rat models with either normal pancreas (group A), PanIN (group B, PanIN-2), or PC (group C) were established, and three samples per group were collected. Approximately 300 mg of each tissue sample was divided into 3-mm^3 pieces and then homogenized with a Dounce's homogenizer on ice in 1 mL lysis solution containing 7 mol/L urea, 2 mol/L thiourea, 4% CHAPS and a protease inhibitor mixture (Bio-Rad). The sample lysates were then placed into Eppendorf tubes and sonicated on ice for 10 s, and this procedure was repeated eight times. The sample lysates were centrifuged at 14000 rpm for 60 min, and the supernatants were collected. The total protein concentration in each sample was determined by the Bio-Rad method.

The protein concentration in each sample was dissolved to 5 μg/μL with lysis buffer, and equal amounts of protein from each individual sample were pooled to make the internal standard. The pH of all the samples and the internal standard was adjusted to 8.0-9.0. Five DIGE gels were included in the experimental design, as shown in Table 1. Two different samples in each gel contained 50 μg protein labeled with 1 μL green (Cy3) or red (Cy5) fluorescent dyes (Amersham Biosciences). The third sample contained 50 μg internal standard labeled with yellow (Cy2) fluorescent dye. The labeling reaction was performed on ice for 30 min in the dark and quenched with 1 μL of 10 mmol/L lysine for 10 min.

One-dimensional isoelectric focusing was carried out on an Amersham Biosciences Ettan IPGphor IEF system. The samples (100 μg) were loaded using 13-cm pH-3-10 IPG strips. Isoelectric focusing was performed at 30 V for 12 h, 500 V for 1 h, 1000 V for 1 h, 8000 V for 8 h and 500 V for 4 h. After IEF, the IPG strips were equilibrated with an equilibration buffer containing 50 mmol/L Tris-HCl, 6 mol/L urea, 30% glycerol, 2% SDS, and 1% DTT for 15 min at room temperature, followed by 2.5% iodoacetamide instead of 1% DTT in equilibration buffer for another 15 min. The equilibrated strip was transferred to the top of 1 mm of a 12.5% SDS-polyacrylamide gel and

fixed with 0.5% agarose. The second dimension of SDS-PAGE was performed at a constant current of 15 mA/gel for 30 min and 30 mA/gel until the bromphenol blue fronts reached 0.5 cm from the bottom of the gel.

The fluorescent dye-labeled proteins in each gel were scanned with a Typhoon 9400 fluorescence scanner (Amersham Biosciences) at different wavelengths specific for Cy2 (488/520 nm), Cy3 (532/580 nm) and Cy5 (633/670 nm). Image analysis of 2D-DIGE was performed with Decyder 5.0 software (GE Healthcare) according to the manufacturer's recommendations. Differential protein analysis of the three groups was carried out using one-way ANOVA. Differentially expressed protein spots ($P < 0.01$) were marked.

Protein identification

Two-dimensional electrophoresis of preparative gels containing 1 mg protein was performed like 2D-DIGE, and the gels were stained with Coomassie brilliant blue. The protein spots to be identified were manually excised from the preparative gels. The gel pieces were de-stained in 3% acetic acid and 100 mmol/L ammonium bicarbonate, and in-gel digestion was performed for 20 h at 37℃ in 40 mmol/L ammonium bicarbonate containing 0.01 μg/μL trypsin. The tryptic peptides were extracted with 0.1% TFA (60% ACN from the supernatants), and the extracts were lyophilized and saved at -80℃.

The lyophilized peptide mixtures were re-dissolved in 0.1% TFA, the peptide solution was washed with 0.1% TFA, and the 60% ACN was mixed with HCCA (5 mg/mL). Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) and MALDI-TOF/TOF mass spectrometry were obtained using a Bruker-Daltonics Autoflex TOF-TOF Lift mass spectrometer. Peptide mass fingerprints (PMFs) were acquired for each differentially expressed protein in positive-reflection mode (20 kV accelerating voltage, 23 kV reflecting voltage). The PMFs were searched in the database of the National Center for Biotechnology Information (NCBI) of Rattus using the software Mascot. Carbamidomethyl was specified as fixed modification, and the oxidation as variable modifications, and the peptide mass tolerance was ± 100 ppm. The statistically significant protein scores were found ($P < 0.05$), and, if more than one protein was identified, the single protein with the top score was matched to the protein spot.

Immunohistochemistry

Three pairs of rat normal pancreas and DMBA-induced PC tissues and 30 pairs of PC and adjacent non-carcinoma tissues from humans in a tissue microarray (Shanghai Outdo Biotech Co. Ltd, OD-CT-DgPan03-002, 19 male and 11 female) were used for immunohistochemisical staining. All of the samples were formalin-fixed and embedded in paraffin. The sample sections were deparaffinized and successively rehydrated in xylene and alcohol and washed three times in phosphate buffered saline (PBS). The endogenous peroxidase activity was blocked with 3% hydrogen peroxide in PBS for 10 min, and the sections were then incubated with 0.05% trypsin for 30 min at 37℃. After three washes with PBS, nonspecific binding was blocked with 10% BSA in PBS for 30 min at 37℃. The sections were then incubated overnight at 4℃ with an enolase 1 antibody at a dilution of 1:400, and sections incubated with PBS instead of the enolase 1 antibody, served as control. DAB (1:50) was used to detect the enolase 1 with the deposition of a brown reaction product in the nuclei and cytoplasm of the positive cells. The proportion of positively stained cells in each section was averaged from three high-magnification images.

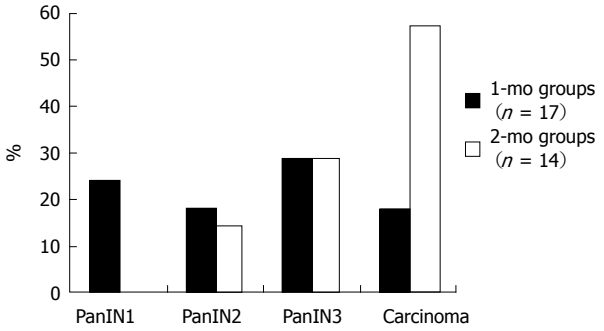

Figure 1 Pathological diagnoses in 1- and 2-mo groups after 7,12-dimethylbenzanthracene implantation. The difference between two groups was statistically significant ($P = 0.0488$), and there was an increase in the frequency of adenocarcinomas in the 2-mo group compared with the 1-mo group ($P = 0.0309$). PanIN: Pancreatic intraepithelial neoplasia.

Statistical analysis

Differences between groups were analyzed by two-tailed Chi-square tests and two-tailed Fisher's exact tests. The significance level was defined as $P < 0.05$.

RESULTS

Rat models of DMBA-induced PanIN and PC

In the 1-mo group, 15% (3/20) of the rats died in the postoperative period, whereas 30% (6/20) died in the 2-mo group. No statistically significant difference in the death rate was observed between the groups ($P = 0.4489$). Pathologic evaluation revealed that 71% (12/17) of the rats had PanIN lesions and 18% (3/17) of the rats had adenocarcinomas in the 1-mo group. Of these, 24% (4/17) had PanIN1 lesions, 18% (3/17) had PanIN2 lesions and 29% (5/17) had PanIN3 lesions. In the 2-mo group, 14% (2/14) had PanIN2 lesions, 29% (4/14) had PanIN3 lesions, and 57% (8/14) had adenocarcinomas (Figure 1). The difference in the proportions of neoplastic changes was statistically significant between the two groups ($P = 0.0488$), and there was an increase in the frequency of adenocarcinomas in the 2-mo group compared with the 1-mo group ($P = 0.0309$). No neoplastic changes were observed in any of the animals in the normal group.

2D-DIGE analysis of differential proteomic expression of DMBA-induced PanIN and PC

After spot quantification and statistical analysis using the 2D-DIGE approach described above, 1445, 1469, 1380,

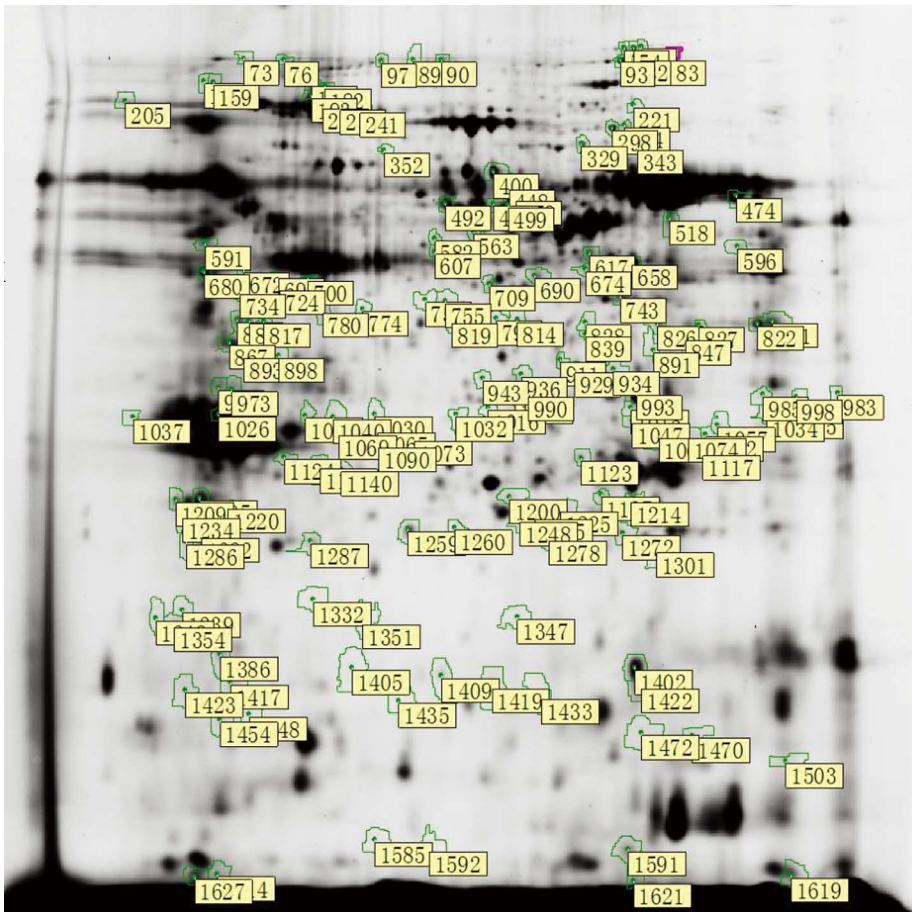

Figure 2 Map of 155 protein spots with significantly differential expression ($P < 0.01$) in normal pancreatic (group A), pancreatic intraepithelial neoplasia-2 (group B) and pancreatic carcinoma (group C) tissues. Thirty-one protein levels progressively increased, and 17 protein levels progressively decreased from normal pancreatic tissue to 7,12-dimethylbenzanthracene-induced pancreatic intraepithelial neoplasia and pancreatic carcinoma.

1512, and 1637 spots were identified in gel1, gel2, gel3, gel4 and gel5, respectively. After background subtraction and radiometric normalization, matched spots from all of the gels were used for statistical analysis. By using the criterion of $P < 0.01$, 155 spots were significantly differentially expressed by their relative abundances in groups A, B and C (Figure 2). Furthermore, 31 protein levels progressively increased from normal pancreas to pancreas with DMBA-induced PanIN and PC. Additional 17 protein levels progressively decreased, and 21 spots were selected for identification using a MALDI-TOF/TOF mass spectrometer. We found that the abundances of enolase 1, pancreatic elastase 3B (ELA3B), necdin, Hbp23, chromodomain helicase DNA-binding protein 3 (CHD3), heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNP A2/B1), retinoid X receptor-interacting protein (Rap80), guanine nucleotide-binding protein (G protein), and beta polypeptide 2-like 1 (Gnb2l1) progressively increased with the severity of the disease. The abundances of carboxyl ester lipase (CEL), tumor protein translationally controlled 1 (TPT1), expressed in non-metastatic cells 2 (NME2), phosphoenolpyruvate carboxykinase 2 (PCK2), an unnamed protein product, and glycine C-acetyltransferase progressively decreased with DMBA-induced disease severity (Table 2).

Immunohistochemical analysis of enolase 1

Immunohistochemical staining showed that the expression level of enolase 1 was highest in the nucleus and cytoplasm; membranous immunoreactivity was also detected. The enolase 1 expression levels in DMBA-induced PC and normal rat pancreatic tissues were compared, and the staining results showed that the rat PC tissue had a higher enolase 1 expression level (P = 7.5633E-014). In the rat PC tissue, an average of 83% of cells expressed enolase 1, whereas only 31% of the cells in rat normal pancreatic tissues expressed enolase 1. The expression of enolase 1 was compared in pancreatic cancer and adjacent non-carcinoma tissues from 30 patients, and the average proportion of enolase 1-positive cells in the human pancreatic cancer tissue was 91%, and only 37% in adjacent non-carcinoma tissues. The expression of enolase 1 was also significantly increased in human PC tissues (P = 3.3514E-015) (Figure 3).

DISCUSSION

Animal models of PanIN and PC that resemble the human disease are very important for research. The characteristics of these models should include the following: (1)

Table 2 Differentially expressed proteins identified by matrix-assisted laser desorption/ionization-time of flight/time of flight tandem mass spectrometry in normal pancreatic, pancreatic intraepithelial neoplasia-2 and pancreatic cancer tissues

Spot position	Protein number in NCBI	Protein name	MW	PI	Mascot PMF score	*P*
230	Mixture,gi|6753406 + gi|74205924	Carboxyl ester lipase+unnamed protein product	70	6	136	4.10E-5
780	gi|158186649	Enolase 1	40	5.5	76	5.40E-5
1205	gi|6678437	Tumor protein translationally controlled 1	25	5	98	7.90E-5
1402	gi|55926145	Expressed in non-metastatic cells 2	20	8	185	0.000
1132	gi|149024340	Pancreatic elastase 3B	25	5.5	91	0.000
1066	gi|56676354	Necdin	30	8	63	0.000
1272	gi|6435547	Hbp23	25	7.5	62	0.001
226	gi|6753406	Carboxyl ester lipase	90	5.5	118	0.001
1405	gi|109488364	Chromodomain helicase DNA-binding protein 3	20	5.5	72	0.001
755	Mixture,gi|149033753 + gi|149039895	Albumin+retinoid X receptor-interacting protein	40	6.5	181	0.001
1259	gi|12832572	Unnamed protein product	25	6	38	0.001
298	gi|149063967	Phosphoenolpyruvate carboxykinase 2	66	7.5	229	0.002
1057	gi|4504447	Heterogeneous nuclear ribonucleoprotein A2/B1	30	8.5	62	0.002
993	gi|5174447	Guanine nucleotide binding-protein (G-protein), beta polypeptide 2-like 1	30	7.5	146	0.003
1040	gi|5174447	Guanine nucleotide binding-protein (G-protein), beta polypeptide 2-like 1	30	5.5	142	0.004
658	gi|66730435	Glycine C-acetyltransferase	40	8	211	0.008

NCBI:National Center for Biotechnology Information; PMF:Peptide mass fingerprint; MW: Molecular weight ; PI: Isoelectric point.

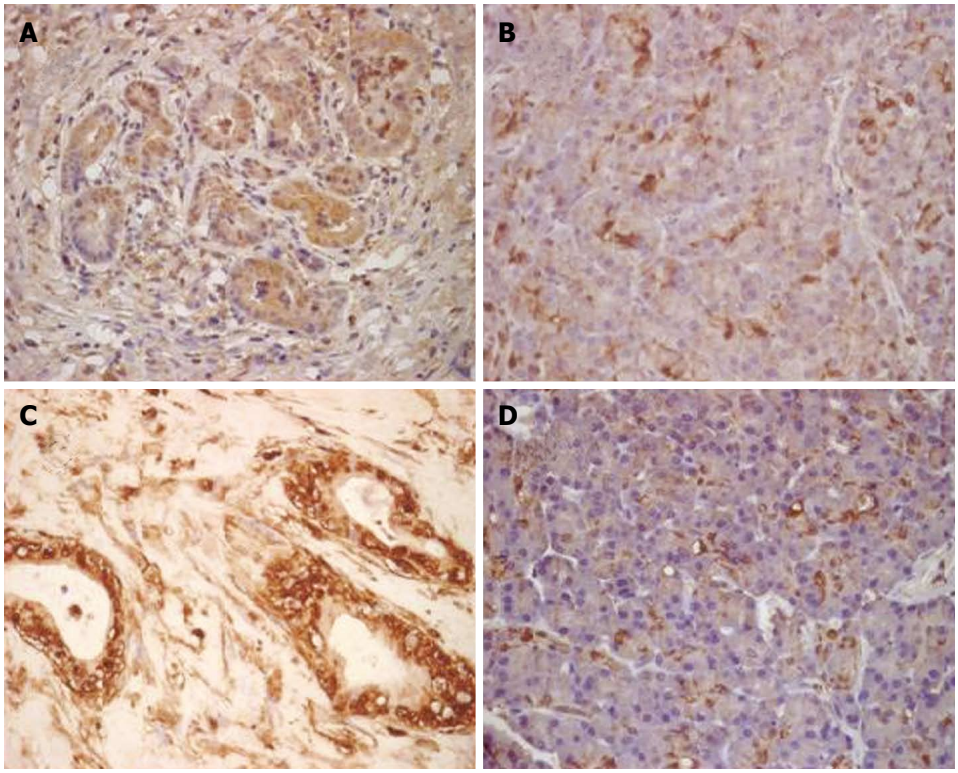

Figure 3 Enolase 1 expression was significantly increased in human and rat pancreatic carcinoma (immunohistochemistry, magnification × 400). A: Positive expression in rat pancreatic cancer; B: Weakly positive expression in the normal rat pancreas; C: Positive expression in human pancreatic cancer; D: Weakly positive expression in human adjacent non-carcinoma tissues.

A pancreatic ductal phenotype that arises from pancreatic ductal cells; (2) Extensive reactive proliferation of the

connective tissue; (3) Obstruction of the bile duct and stomach during disease progression; (4) Early neural, peritoneal, and liver metastases; and (5) High rates of K-ras, *P16* and *P53* gene mutations[16]. The experimental models of PanIN and PC in the present study are chemical carcinogenesis models. DMBA-induced pancreatic carcinogenesis models resembled human PC in several previous studies. Rivera *et al*[6] found that DMBA was one of the three different carcinogens reliably producing PC histology that is similar to human ductal adenocarcinoma. DMBA was either implanted directly into the pancreas or infused into the pancreatic ducts of SD rats, and the development of ductal hyperplastic, atypical, and dysplastic changes preceding and accompanying the invasive pancreatic ductal adenocarcinoma could be observed in this experimental model. Jimenez *et al*[7] demonstrated that DMBA-induced rat tubular complexes and pancreatic adenocarcinomas strongly expressed ductal cell markers (keratin, cytokeratins 19 and 20) but not acinar cell markers (chymotrypsin), suggesting that these tumors arose from ductal cell transformation. K-ras mutations were significantly more frequent in DMBA-induced PC tissues than in normal tissues, with a prevalence of up to 91%[8]. Pancreatic carcinogenesis induced by DMBA implantation in mice was re-evaluated according to the PanIN classification system after its establishment, and extensive pathological changes characteristic of PanIN could be observed one month after carcinogen implantation[9]. We used the PanIN classification system in the histological analysis, and our pathologic evaluation showed that 71% (12/17) of the rats had PanIN lesions and 18% (3/17) had adenocarcinomas in the 1-mo group. In the 2-mo group, 43% (6/14) had PanIN lesions and 57% (8/14) had adenocarcinomas. The frequencies of PanIN and PC in our rat models were similar to a previous study in mice[9]. Our models were satisfactory because the development of precursor lesions (PanIN) and an invasive adenocarcinoma were observed in all groups. There was an increase in the frequency of adenocarcinomas, and the PanIN classification increased with DMBA induction, reflecting the dynamic development of a normal pancreas into a cancerous pancreas.

There are several techniques available for protein separation, both gel- and non-gel-based. Gel-based techniques include traditional two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and, more recently, 2D-DIGE. 2D-DIGE usually contains three samples labeled with three distinct fluorescent dyes: Cy2, Cy3 and Cy5. The Cy2 dye is typically used to label an internal standard; the strength of the internal standard helps map the spots/proteins between the gels, thus rendering different gels more comparable[17-19]. In the current study, we used 2D-DIGE and MALDI-TOF/TOF tandem mass spectrometry to profile pancreatic proteins in rat models of DMBA-induced PanIN and PC and compared these profiles with those of normal rats. Our 2D-DIGE data showed that 155 spots were significantly differentially expressed based on their relative abundances in the three groups. Of these, 31 protein levels progressively increased from normal to PanIN and then to PC tissue, whereas 17 protein levels progressively decreased. These results demonstrated synchronous dynamic changes in the pancreatic protein expression profile after DMBA induction. A number of novel proteins were identified that may be involved in important aspects of mRNA transcription, DNA damage repair, chromatin remodeling, oxidative stress, regulation of tumor growth and metastasis, glucose metabolism, and the synthesis and secretion of pancreatic enzymes.

Enolase 1, also known as α-enolase or non-neuronal enolase (NNE), is an isoenzyme of enolase, which is a glycolytic enzyme catalyzing the conversion of 2-phosphoglycerate into phosphoenolpyruvate. Recent researches have shown that enolase 1 plays an important role in several biological and pathophysiological processes. Enolase 1 is thought to play a potential role in tumorigenesis, cancer invasion and metastasis. Previous proteomic studies reported that enolase 1 was up-regulated in several cancers, such as hepatocellular carcinoma[20-22], non-small lung cancer[23,24], esophageal adenocarcinoma[25], prostate cancer[26], colon cancer[27], oral epithelial and squamous cell carcinoma[28]. In our study, we found a significant up-regulation of enolase 1 in rat models of DMBA-induced PanIN and PC with further verification by the immunohistochemical analysis of human and rat tissues. These results agree with other proteomic researches on human PC. Mikuriya *et al*[29] found that the expression levels of glycolytic enzymes, including enolase 1, increased in the cancerous pancreatic tissues of 10 patients compared with the paired non-cancerous tissues, as determined by proteomic profiling using two-dimensional electrophoresis and liquid chromatography-mass spectrometry/mass spectrometry. Shen *et al*[30] used a proteomic approach using two-dimensional gel electrophoresis and mass spectrometry to identify differentially expressed proteins in six PC cases, two normal adjacent tissues, seven cases of pancreatitis, and six normal pancreatic tissues. Alpha-enolase was also specifically overexpressed in tumors compared with normal and pancreatic tissues.

The hnRNP A2/B1 protein was shown to be up-regulated in our models. This protein plays an important role in the biogenesis and transport of mRNA. The over-expression of hnRNP A2/B1 indicates that normal transcriptional regulation is altered. A previous study also found high levels of hnRNP A2/B1 expression in a limited number of human pancreatic adenocarcinomas from smokers and two pancreatic tumor cell lines, HPAF-11 and SU 86.86[31]. CEL is one kind of pancreatic exocrine enzyme, and we found that it was down-regulated after DMBA induction. Reuss *et al*[32] verified strong CEL gene expression in the acinar cells of the normal pancreas, and adenocarcinomas showed no expression, which agrees with our results. The other differentially expressed proteins that we identified are rarely reported in PC, and their relationships with PC await further clarification.

In summary, our data have shown DMBA implantation into the pancreas is an effective method to establish rat models of PanIN and PC, and the protein changes observed in DMBA-induced PanIN and PC, such as enolase 1 up-regulation, demonstrate the feasibility of

identifying potential molecular targets for the early diagnosis and treatment of PC.

COMMENTS

Background

The early detection of pancreatic cancer (PC) is still difficult, but proteomic techniques are emerging as important tools to find potential biomarkers and new therapeutic targets for PC and precancerous pancreatic lesions.

Research frontiers

7,12-dimethylbenzanthracene (DMBA) implantation into the pancreas is known to induce PC and pancreatic ductal intraepithelial neoplasia (PanIN) in mice and rats that resemble human PC with K-ras gene mutations and the activation of Notch signaling.

Innovations and breakthroughs

Precancerous pancreas lesions and the early stages of PC among clinical diagnoses are rarely found. In the present research, the authors studied the proteomic variabilities of rat models of DMBA-induced PanIN and PC.

Applications

The protein changes in DMBA-induced PanIN and PC reported in this article, such as enolase 1 up-regulation, demonstrate the feasibility of identifying potential molecular targets for early PC diagnostics and therapeutics.

Terminology

Two-dimensional fluorescence difference gel electrophoresis is a gel-based technique that has recently been used for protein separation. It contains three samples labeled with three distinct fluorescent dyes: Cy2, Cy3 and Cy5.

Peer review

This is a good study to define the stages of pancreatic carcinoma in experimental models and its correlation with various markers.

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