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Early changes in the urine proteome in a diethyldithiocarbamate-induced chronic pancreatitis rat model

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ABSTRACT

Without homeostasis mechanisms, urine accumulates early changes in biomarkers and can be a better and earlier biomarker source than blood, especially for chronic diseases. This study tests whether early changes can be detected in a rat model of chronic pancreatitis induced by intraperitoneal injection of diethyldithiocarbamate. Urinary proteins from three rats were profiled by liquid chromatography coupled with tandem mass spectrometry. Compared with before injection, fifty differential proteins that had human orthologs were significantly changed in the chronic pancreatitis rats. At week 2, fifteen differential proteins were identified when no obvious pathological changes had yet appeared. Among them, twelve proteins were altered at the other two time points, five had previously been associated with chronic pancreatitis. Inflammatory cytokines infiltration, acinar disruption and fibrosis were detected at week 3 and week 4, at which points fourteen identified differential proteins had been reported to be differentially expressed in the serum or pancreatic tissue of chronic pancreatitis patients and other animal model studies. These include proteins that are significant in acute-phase response (FETUA, F2, FINC and REG3G), extracellular matrix organization (COMP, FINC) and tissue remodeling (CSPG4, GAS6). REG3G has been described abnormally expressed in several human digestive system diseases such as chronic pancreatitis.

Significance: This study demonstrate that changes caused by chronic pancreatitis can be reflected early in urinary proteins. New clues for the early diagnosis of chronic pancreatitis can be found even with only the small number of model animals used.

1. Introduction

Biomarkers are measurable changes associated with physiological or pathological processes. Currently, biomarkers are widely used in the early diagnosis or prognosis of diseases. Without homeostasis mechanisms, urine is enriched with the wastes from the body and, thus, can reflect early changes caused by diseases, theoretically providing a better and earlier biomarker source than blood [1].

Chronic pancreatitis (CP) is an irreversible bio-inflammatory disease that eventually leads to loss of exocrine and endocrine function, causing complications such as steatorrhea, loss of weight and diabetes. The main histologic features of CP are chronic inflammation and interstitial fibrosis of the pancreas [2]. There is no widely used diagnostic standard for CP, and early diagnosis is difficult. Abdominal pain is the main clinical symptom, but this is not specific to CP. Imaging and pancreatic function tests are of limited value in the early diagnosis of the disease [3]. At present, treatment of CP can only temporarily relieve pain and complications but cannot cure the disease [4]. Meanwhile, chronic inflammation of the pancreas in CP is a risk factor for the development of pancreatic cancer (PC) [5]. If diagnosed earlier, effective measures may be used to prevent the development of cancer.

Urine is easily affected by gender, diet, medicine and geographical area [6]. Using animal models to study urinary biomarkers can avoid problems stemming from the genetic background and living environment of clinical samples and can be used to monitor the whole process of disease development. Currently, biomarker research using animal models has been conducted to study chronic diseases including obstructive nephropathy [7], pulmonary fibrosis [8], hepatic fibrosis [9], multiple sclerosis [10], myocarditis [11] and glomerular diseases [12], as well as subcutaneous tumors [13] and glioma [14]; the researches have found potential urinary biomarkers in the early stages of diseases through proteomic technology.

Oxidative stress is an important factor in the development of pancreatic fibrosis [15]. Diethyldithiocarbamate (DDC) can inhibit

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superoxide dismutase activity, thereby increasing lipid peroxides, causing progressive pancreatic injury and repeated oxidative stress leading to pancreatic fibrosis. Repeated administration of DDC to cause pancreatic fibrosis produces a model of CP with similar pathological process and pathological features to those of human CP [16]. In this study, we used liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) to analyze the urinary proteome in DDC-induced CP rats. Our aim was to screen for differential proteins associated with disease progression to provide some clues for the early biomarkers of CP.

2. Materials and methods

2.1. Rat model of chronic pancreatitis

Thirty-eight male Wistar rats (200–220 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The animal license was SCXK (Beijing) 2016-0011. The experiment in this study was approved by Institutional Animal Care Use & Welfare Committee of Institute of Basic Medical Sciences, Peking Union Medical College (Animal Welfare Assurance Number: ACUC-A02-2014-007). All animals were housed under a standard 12 h light/12 h dark cycle and in a standard environment (room temperature 22 ± 1 °C, humidity 65% - 70%). Diethyldithiocarbamate was purchased from Sigma.

Wistar rats were randomly divided into two groups: the control group (n = 12) and the experimental group (n = 26). The DDC-induced CP rat model was established as follows [16]. Rats in the experimental group were given an intraperitoneal injection of 500 mg/kg body weight of DDC. Rats in the control group were given an intraperitoneal injection of 500 mg/kg body weight of normal saline. The injection site was in the right lower quadrant. The experimental and control groups were injected twice per week for six weeks.

2.2. Histological analysis of chronic pancreatitis rats

Four rats in the experimental group and two rats in the control group were randomly sacrificed at 2 weeks, 3 weeks, 4 weeks and 6 weeks after injection. Pancreatic tissue samples were quickly fixed in 10% formalin. Then, the samples were embedded in paraffin, sectioned, and evaluated with hematoxylin and eosin (H&E) staining and Masson's trichrome staining.

2.3. Urine collection and sample preparation

Urine samples were collected from the experimental group at weeks 0, 2, 3 and 4. Without any treatment, rats were individually placed in metabolic cages overnight naturally for 10 h to collect urine samples. During urine collection, no water or food was provided for rats to avoid urine contamination. After collection, urine samples were quickly stored at −80 °C until the sample preparation of LC-MS/MS analysis was conducted.

Urine samples were centrifuged at 12,000*g* for 30 min at 4 °C to remove impurities and large cell debris. The supernatants were precipitated with three volumes of pre-chilled ethanol at −20 °C for 2 h and then centrifuged at 12,000*g* for 30 min. The resulting pellet was resuspended in lysis buffer (8 mol/L urea, 2 mol/L thiourea, 50 mmol/L Tris, and 25 mmol/L DTT) and then centrifuged at 12,000*g* for 30 min at 4 °C. The supernatant containing the resulting protein extract was placed into a new tube. The Bradford assay was used to determine the protein concentration.

The urinary proteins at weeks 0, 2, 3 and 4 were digested using the FASP method [17]. Each 100 μg of protein was added to a 10 kDa filter device (Pall, Port Washington, NY, USA). After sequentially washing with UA (8 mol/L urea, 0.1 mol/L Tris-HCl, pH 8.5) and 25 mmol/L NH_4HCO_3, the proteins were reduced with 20 mmol/L dithiothreitol (DTT, Sigma) at 37 °C for 1 h and then alkylated with 50 mmol/L iodoacetamide (IAA, Sigma) in the dark for 30 min. Then, the samples were centrifuged at 14,000*g* for 30 min at 18 °C, followed by washing once with UA and three times with 25 mmol/L NH_4HCO_3. The samples were digested with trypsin (enzyme-to-protein ratio of 1:50) at 37 °C overnight. The peptide mixtures were collected and desalted using Oasis HLB cartridges (Waters, Milford, MA) and then dried by vacuum evaporation and stored at −80 °C.

2.4. LC-MS/MS analysis

The digested peptides were separated by EASY-nLC 1200 HPLC system (Thermo Fisher Scientific, USA). First, the digested peptides were acidified with 0.1% formic acid, and the peptide concentration was determined by the BCA assay, then diluted to 0.5 μg/μL. Then, 1 μg of peptides was loaded onto a trap column (Acclaim PepMap®100, 75 μm × 100 mm, 2 μm, nanoViper C18). The elution time was 60 min, and the column flow rate was 0.3 μL/min. The elution gradient was 5% to 40% mobile phase B (mobile phase A: 0.1% formic acid; mobile phase B: 89.9% acetonitrile). Mass spectrometry analysis was performed using a Thermo Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific, USA) [18]. MS data were collected in a data-dependent type with the orbitrap as the detector. Survey MS scans were acquired by the Orbitrap in a 350–1550 m/z range with the resolution set to 120,000. For the MS/MS scan, the resolution was set at 30,000 and the HCD collision energy was chosen at 30. Dynamic exclusion was employed with a 30 s window.

Twelve urine samples from three randomly selected DDC-induced CP rats at four time points (weeks 0, 2, 3, and 4) were chosen for MS analysis. Two technical replicate analyses were performed for each sample.

2.5. Data analysis

Label-free quantification was performed using the Progenesis QI LC-MS software (version 4.1, Nonlinear, UK). All MS data were searched using Mascot Daemon software (version 2.5.1, Matrix Science, UK) with the SwissProt_2017_02 database (taxonomy: Rattus; containing 7992 sequences). The search conditions were set as follows: trypsin digestion was selected, 2 sites of leaky cutting were allowed, cysteine was fixedly modified, methionine oxidation and protein N-terminal acetylation were mutagenic, the peptide mass tolerance was set to 10 ppm and fragment mass tolerance was set to 0.05 Da.

Statistical analysis that compare between four time points was performed by one-way ANOVA. The proteins identified at weeks 2, 3, and 4 were compared with those at week 0. The differential proteins were screened with the following criteria: proteins with at least two unique peptides were allowed; fold change in increased group ≥2 and fold change in decreased group ≤0.5; $P < 0.05$ by independent sample *t*-test. Group differences resulting in $P < 0.05$ were identified as statistically significant. All results are expressed as the mean ± standard deviation.

2.6. Functional annotation of the differential proteins

DAVID 6.8 (https://david.ncifcrf.gov/) was used to perform the functional annotation of the urinary differential proteins identified at weeks 2, 3 and 4. The detailed annotation included the biological process, cellular component and molecular function.

3. Results and discussion

3.1. Characterization of DDC- induced rats

Rats in the control group had normal body weights, while rats in the experimental group injected with DDC showed a slow growth of body weight (Fig. S1). There was a significant difference in body weight