Two-dimensional electrophoresis for comparative proteomic analysis of human bile

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BACKGROUND: **Proteomic analysis of bile fluid holds promise as a method to identify biomarkers of bile tract diseases, especially for tumors. Two-dimensional electrophoresis (2-DE) is a popular and proven separation technique for proteome analysis, but using this strategy for bile fluid analysis is still not fully developed. This study was undertaken to (a) establish a reliable method for general clean-up to make bile fluid samples suitable for 2-DE; (b) obtain 2-D biliary maps with high reproducibility and resolution; and (c) identify protein patterns present in 2-D biliary maps for potential tumor biomarker discovery, with the intention of distinguishing malignant from benign causes of bile duct obstruction.**

METHODS: **Bile fluid samples were obtained from two patients suffering from malignant and benign bile tract obstruction (one patient with cholangiocarcinoma as the experimental case, the other with cholelithiasis as control). A variety of sample preparation options, including delipidation, desalination and nucleic acid removal, were adopted to remove contaminants that affect 2-DE results. After that, each 350 μg purified sample was loaded onto nonlinear IPG strips (18 cm, pH 3-10 and pH 4-7) for first-dimension isoelectric focusing, and 12.5% SDS-PAGE electrophoresis for second dimension separation. Then 2-D maps were visualized after silver staining and analyzed with the Image Master 2-D software.**

RESULTS: **A large number of protein spots were separated in 2-D maps from the experimental and control groups, with means of 250 and 216 spots on pH 3-10 IPG strips, and 182 and 176 spots on pH 4-7 strips, respectively. Approximately 16 and 23 spots were differentially expressed in matched pairs from the experimental and control cases using pH 3-10 and pH 4-7 strips.**

CONCLUSIONS: **This study established a reliable sample preparation process suitable for 2-DE of bile fluid. By this method, 2-D biliary maps with high reproducibility and resolution were obtained. The differentially displayed proteomes in the 2-D biliary maps from the experimental and control groups indicated the potential application for bile fluid analysis to identify disease-associated biomarkers, especially for biliary tract tumors.**

(***Hepatobiliary Pancreat Dis Int 2007; 6: 402-406***)

KEY WORDS: **proteome;**
electrophoresis, gel, two-dimensional;
bile

Introduction

Biliary tract tumors have proven challenging to treat and manage due to their poor sensitivity to conventional therapies and our inability to prevent or to detect early tumor formation. Major improvement in the survival of patients with cancers of the biliary tree will probably not result from more aggressive or advanced surgical techniques or oncologic radiation therapy. Instead, efforts should be made for prevention, early detection, and novel treatments derived from basic research. There is a need for new, cost-effective screening methods, including simple assays for tumor markers in the serum, bile or stool.[1-4]

In recent years, genomic and transcriptomic technologies have been widely used for investigating cell function and disease mechanisms, but proteomics offers the opportunity to answer biological questions we never thought possible.[5, 6] Among many different

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strategies for proteomic analysis, two-dimensional electrophoresis (2-DE) is a powerful and widely used method for the complex protein mixtures extracted from cells, tissues, or other biological samples.[7-10] This technique sorts proteins according to two independent properties in two discrete steps: the first-dimension step, isoelectric focusing (IEF), separates proteins according to their isoelectric points (pI), while the second-dimension step, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), separates proteins according to their molecular weights (Mr, relative molecular weight). Each spot on the resulting 2-D array corresponds to a single protein species in the sample. Thousands of different proteins can thus be separated, and information such as the protein pI, the apparent molecular weight, and the amount of each protein is obtained. With 2-DE for comparative proteomic analysis, many much more sensitive and specific tumor biomarkers have been identified from biofluids (e.g. cerebrospinal fluid, urine and nipple aspirate fluid),[11-13] but human bile analysis is still not fully developed. Salts, lipids, nucleic acids and other contaminants, which often abound in bile fluid, dramatically affects both reproducibility and resolution of 2-DE. The objectives of our study were to establish a reliable sample preparation method and 2-DE options suitable for comparative proteomic analysis of bile fluid. In addition, we attempted to identify different protein patterns present in 2-D biliary maps for discovery of biliary disease-associated biomarkers, with the intention of distinguishing malignant from benign causes of bile duct obstruction.

Methods

Sample preparation

Bile fluid samples were obtained during surgical drainage procedures from two patients suffering from malignant or benign bile tract obstruction (one with cholangiocarcinoma as the experimental case, of the other with cholelithiasis as control). A total volume of 10 ml crude bile fluid was collected from each patient by T-tube 3 days after surgical external drainage, and immediately delivered to the lab on dry ice without any protease inhibitors. The samples were sonicated and centrifuged at 16 000 g for 15 minutes at 4 ℃ to remove debris, nucleic acid and mucins as a preliminary separation. For sample delipidation, each 1 ml of preliminary separated sample was then mixed with 250 μl of Cleanascite™ HC (Ligo-Chem, Inc., Fairfield, NJ, USA) followed by rotation for 1 hour at 4 ℃. After incubation, each sample was centrifuged at 16 000 g for 1 minute to clear away the formed lipid-micelles, and the supernatant was transferred to a new tube. In order to reduce the salt concentration and other contaminants, a commercially available microcentrifuge filtration device (YM-3, molecular mass cut-off at 3 kD; Millipore, Bedford, MA, USA) was used to wash away contaminating species. After sample purification, protein concentration was determined with a BCA Kit (Pierce, Rockford, IL, USA).

One-dimensional SDS-PAGE electrophoresis

To validate the effect of sample purification and quantization of protein concentration, one-dimensional SDS-PAGE electrophoresis (5%-10% gradient gel, 0.5 mm thick) was performed before 2-DE. Each 25 μg of sample was mixed with 5×loading buffer, heated at 95 ℃ for 5 minutes and loaded in SDS-PAGE gel for one-dimensional electrophoresis. The samples were separated at 10 mA and 85 V for 4 hours and visualized with colloidal Coomassie blue staining according to the reported methods.[14]

Two-dimensional electrophoresis

For 2-DE, each 350 μg of purified sample was exchanged with lysis buffer (40 mmol/L Tris-HCl, 7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 40 mmol/L DTT) compatible with IEF using the microcentrifuge filtration device. After dilution to 400 μl with rehydration buffer (8 mol/L urea, 2% CHAPS, 0.5% IPG buffer, 0.002% bromphenol blue), the samples were loaded onto IPG strips (18 cm, covering the pH ranges 3-10 and 4-7; Amersham Biosciences, USA) for isoelectric focusing using a Ettan IPG phor Ⅱ (Amersham). After rehydration at 30 V for 12 hours, the voltage/time profile used was as follows: 200 V for 1 hour, 500 V for 1 hour, 1500 V for 30 minutes, 4000 V for 1 hour, and it was held at 8000 V to a total of 60 kVh. After equilibration with a solution containing 6 mol/L urea, 30% glycerol, 2% SDS, 50 mmol/L Tris-HCl, 1% DTT, and 0.002% bromphenol blue, IPG strips were equilibrated again with the same solution containing 4% iodoacetamide instead of DTT. IPG strips were then placed on top of 12.5% homogeneous polyacrylamide gels for second dimension separation under standard conditions at 5 W/gel for 30 minutes followed by 60 W total for 10 hours with a peltier-cooled Ettan DALT (Amersham).

Protein visualization and image analysis

Following 2-DE, silver staining was performed.

Gels were scanned on an image scanner (Amersham) at 400 dots per inch, and spot detection and quantification were performed with Image Master 2-D software (Amersham). The volume of each spot (integrated optical density) was calculated as the product of spot area and spot intensity. The background subtraction was made in a rectangle that completely enclosed each spot, with a local correction mode. To take account of experimental variations, 2-D gels were normalized by dividing each spot volume by the total volume of the matched spots in the 2-D gel image to obtain a normalized spot volume.

Results

Sample preparation and purification

The results of one-dimensional SDS-PAGE electrophoresis validated the effect of sample purification and quantization of protein concentration. When improved strategy was used, the purified bile fluid provided a satisfactory one-dimensional SDS-PAGE map without the smearing of lipid and other contaminants (Fig. 1). The similar abundances of proteins present in each lane also validated the quantization of protein concentration.

Resolution and reproducibility of 2-DE of human bile

After the efficient clean-up of bile fluid samples, the 2-D maps were of high resolution and reproducibility. A large number of protein spots were present in 2-D maps from the experimental and control cases, with means of 250 and 216 spots on the pH 3-10 strips, and 182 and 176 spots on the pH 4-7 strips, respectively (Fig. 2).

Comparative analysis of 2-D maps

When the narrow-pH-range and wide-pH-range 2-DE were used, there were 63 and 120 matched protein spots matched between the experimental and control cases. The percentage matches were 27.04% (pH 3-10) and 67.04% (pH 4-7), respectively. The degrees of expression of these matched spots were significantly different. Among them, 16 (pH 3-10) and 23 (pH 4-7) had variations of normalized spot volume $\geqslant 0.5$. Besides, 187 and 153 protein spots were detected as exclusively expressed in the experimental and control groups using pH 3-10 IPG strips, while 62 and 56 protein spots were detected as exclusively

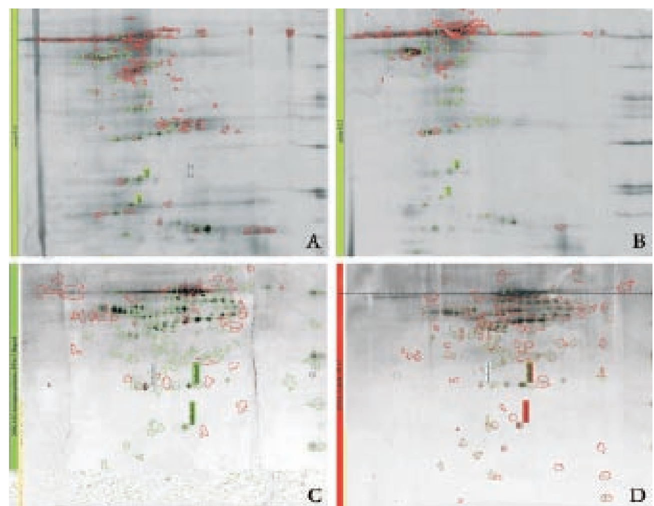

Fig. 2. Proteome separated in the 2-D map of bile fluid between the experimental and control groups using pH 3-10 and pH 4-7 IPG strips. **A:** pH 3-10, experimental case. **B:** pH 3-10, control case; **C:** pH 4-7, experimental case; **D:** pH 4-7, control case. The green outline spots represent the matched pairs. The red outline spots were exclusively expressed in experimental and control groups.

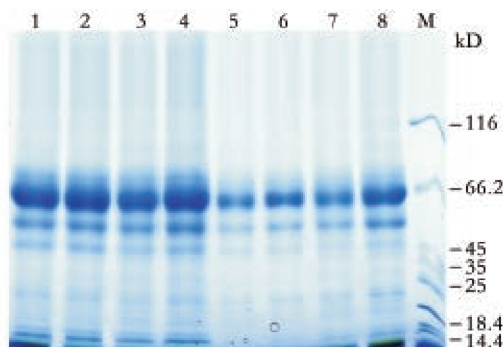

Fig. 1. Colloidal Coomassie blue-stained gel of purified bile fluid samples. Experimental and control groups were repeated for 4 lanes (1-4 for experimental group, 5-8 for control group). M: Marker.

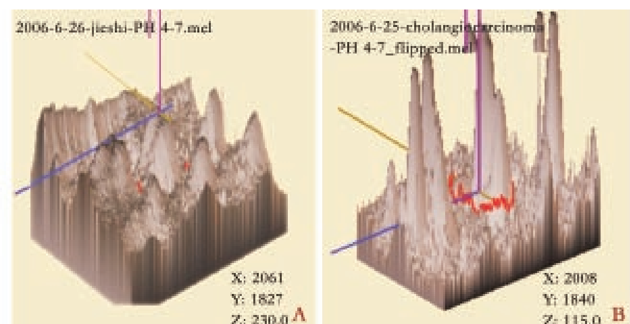

Fig. 3. Representative 3-D images of protein spot variation. Data from a typical matched protein pair. There was a 4.16-fold change in the level of this spot between the experimental (**B**) and control groups (**A**). The variation of V% was 0.612.

expressed in experimental and control groups using pH 4-7 strips (Fig. 3).

Discussion

Proteomic research of biofluid samples makes it possible to screen for diagnostic markers of disease and search for new targets of drug treatment.[15, 16] Disease-related biological information existing in biofluids is useful for dynamic monitoring analysis. Bile is stored, concentrated and transported in the biliary tract after being secreted by hepatocellular and biliary epithelial cells, so abundant biological information related to diseases of the biliary tract can be detected in it. The first comprehensive proteomic analysis of human bile was reported.[17, 18] One-dimensional gel electrophoresis and lectin affinity chromatography, followed by liquid chromatography tandem mass spectrometry, identified 87 unique proteins and established a catalog of protein components in pathologically changed bile fluid obtained from a cholangiocarcinoma patient through ERCP. Another proteomic analysis of human bile has been made in China. Using a shotgun proteomic approach, Zhou et al[19] made a large-scale (218 proteins) identification of biliary proteins obtained from a cholesterol stone patient through laparoscopic cholecystectomy. Comparison of their results, the number of identified proteins present in both studies was 27.

The 2-DE strategy is considered the only comparative proteomics method which can separate thousands of proteins simultaneously at high-flux at present.[20, 21] Sample preparation dramatically affects both reproducibility and resolution of 2-DE,[22] so proper preparation of bile sample was essential to our study. A variety of options for general clean-up of biofluid samples include spin-column purification, dialysis purification and protein precipitation and total protein extraction.[23-26] In this study, we optimized the clean-up method of Kristiansen. Besides the delipidation and desalination, we added sonication to decrease the sample viscosity and background smears caused by nucleic acids. We found that this sample preparation effectively removed the salts, lipids, nucleic acids, and other contaminants. After this sample preparation, we established high-resolution 2-D biliary maps. A large number of protein spots were separated in the 2-D biliary maps from cholangiocarcinoma and cholelithiasis cases, with means of 250 and 216 spots separated in pH 3-10 strips, and 182 and 176 spots in pH 4-7 strips, respectively.

After analytical software from bioinformatics was used for comparative analysis of the biliary proteome expression patterns in the experimental (sample from a cholangiocarcinoma case) and control (sample from a cholelithiasis case) groups, 63 and 120 matched protein spots were obtained. Among these matched spots, 16 and 23 were found respectively to have variations of normalized spot volume value $\geqslant$0.5. In addition, 187 and 153, 62 and 56 protein spots were detected as exclusively expressed in the experimental and control groups. These results not only indicated the remarkably different proteome presentation in the bile fluids between the benign and malignant bile duct obstruction caused by cholangiocarcinoma and cholelithiasis, but also confirmed the potential application of bile to identify disease-associated biomarkers, especially for biliary tract tumors.

In conclusion, we have established a reliable method for general clean-up of bile fluid samples, which is suitable for 2-DE, by which we built up 2-D biliary maps with high reproducibility and resolution. After the comparison of the locations and volumes of protein spots between the experimental and control groups, we obtained information about differentially expressed proteins in bile fluid, which provides a foundation for the discovery of biliary tract disease-associated biomarkers.

Funding: This study was supported by a grant from the Hi-Tech Research and Development of Program of China (863) (No. 2002AA214061).

Ethical approval: Not needed.

Contributors: CB proposed the study, wrote the first draft and analyzed the data. All authors contributed to the design and interpretation of the study and to further drafts. ZSQ is the guarantor.

Competing interest: No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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Received November 18, 2006
Accepted after revision June 20, 2007