Original Article

Plasma membrane proteome analysis of the early effect of alcohol on liver: implications for alcoholic liver disease

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In humans, the over-consumption of alcohol can lead to serious liver disease. To examine the early effects of alcohol on liver disease, rats were given sufficient ethanol to develop liver cirrhosis. Rats before the onset of fibrosis were studied in this work. Plasma membranes (PM) of liver were extracted by twice sucrose density gradient centrifugation. The proteome profiles of PM from ethanol-treated rats and the controls were analyzed using two-dimensional gel electrophoresis (2-DE) and isobaric tag for relative and absolute quantitation (iTRAQ) technology. Ethanol treatment altered the amount of 15 different liver proteins: 10 of them were detected by 2-DE and 5 by iTRAQ. Keratin 8 was detected by both methods. Gene ontology analysis of these differentially detected proteins indicated that most of them were involved in important cell functions such as binding activity (including ion, DNA, ATP binding, etc.), cell structure, or enzyme activity. Among these, annexin A2, keratin 8, and keratin 18 were further verified using western blot analysis and annexin A2 was verified by immunohistochemistry. Our results suggested that alcohol has the potential to affect cell structure, adhesion and enzyme activity by altering expression levels of several relevant proteins in the PM. To the best of our knowledge, this is the first time to study the effect of alcohol on the liver PM proteome and it might be helpful for understanding the possible mechanisms of alcohol-induced liver disease.

Keywords alcohol; liver fibrosis; 2-DE-MS; keratin 18; iTRAQ

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Introduction

Alcoholic liver disease (ALD) is one of the leading causes of liver disease and liver-related death worldwide [1]. With continuing alcohol use, the disease progresses via continuing cellular injury, inflammation, impairment of hepatic regeneration, and increasing fibrogenesis to cirrhosis and its complications [2–5].

Alcohol-induced liver disease is very complex. Alcohol or its metabolites may alter plasma membrane (PM), mitochondria, intracellular ion homeostasis, or degradative enzyme activity in hepatocytes or non-parenchymal cells during injury, regeneration, and repair, and stimulate the cell cycle through membrane signal transduction–nuclear pathways [6,7]. It is necessary to simplify experiment process and find some lower abundant biomarkers. During the development process of alcohol-induced liver disease, PM plays an important role. Alcohol can increase the membrane permeability and induce membrane defects [6–8]. The proteins in PM acting as ‘doorbells’ and ‘doorways’ play crucial roles in cell function including intercellular communication, cellular development, cell migration and drug resistance [9–12]. So it is very useful to study the PM proteins for finding new drug targets in the ALD treatment and understanding the mechanism of liver fibrosis development under alcohol induction. However, so far, little is known about the role of cell surface proteins in the development of liver fibrosis and how alcohol may affect them.

Recent advances in high-throughput membrane proteomic methods have the potential to help illuminate mechanisms involved in such alcohol-induced membrane defects and increased permeability [13]. Although great progress
has been made in membrane proteomics [13,14], it is still a challenge to identify membrane proteins due to their hydrophobic properties and low abundance. Independent proteomic studies of alcohol usage [15] and liver fibrosis [16] have, so far, been performed on plasma/serum, unfractionated tissues, or cell lines. Altered levels of proteins, including α-famin, α-1 B glycoprotein and angiotensinogen, were reported in the studies of liver fibrosis [16]. An increased abundance of fibrinogen E chain, apolipoprotein A–I, and other proteins was reported in association with alcohol consumption [15]. However, until now, proteomic research methods have yet to be developed for the investigation of membrane proteins related to liver fibrosis.

Recently, we have built a rat model showing liver fibrogenesis in response to serum or alcohol treatment. PM proteomic study has indicated a broad range of proteins related to the stage of fibrosis (scores 2–4; our published paper [17] and another prepared manuscript: PM proteome analysis of alcohol liver cirrhosis).

In this study, we used multiple proteomic technologies to examine early protein changes in liver PM before the onset of fibrosis. Liver samples, with stage 0 fibrosis, were collected after 2 weeks of in vivo alcohol treatment. A subcellular proteomic method based on two-dimensional gel electrophoresis/mass spectrometry (2-DE/MS) and isobaric tag for relative and absolute quantitation-liquid chromatography-mass spectrometry (iTRAQ-LC-MS) was used to study the differences in protein profiles. Three selected proteins of altered abundance were further identified by western blot as annexin A2 (ANXA2), keratin 8 (K8), and keratin 18 (K18). Immunohistochemistry analysis was used to verify the expression of ANXA2. Further investigation will focus on the nature of these early liver PM proteomic changes, which may yield clues to the molecular mechanism of alcohol-induced liver disease.

**Materials and Methods**

**Animals and treatment**

Forty-eight 8-week-old male Sprague-Dawley rats (180–200 g) were purchased from the Center of Laboratory Animals, Shanghai Public Health Center, Shanghai, China. All of the animal studies followed the relevant national legislation and local guidelines, and were performed at the Center of Laboratory Animals. The animals were housed four per cage in one animal room (temperature 23 ± 2°C, relative humidity 55% ± 5%, and 12 h-light and 12 h-dark cycle) and with unlimited access to food and water. They were allowed to acclimate for 1 week before the experiment began.

Rats were randomly divided into two groups: alcohol-treated and the control. The former received gastric perfusion with a complex of olive oil (2 ml/kg d) and pyrazole (25 mg/kg d) containing 60% ethanol (10 ml/kg d) of analytical grade (Sinopharm Chemical Reagent Co. Ltd, Shanghai, China) for 9 weeks [18]. The controls were perfused with physiological saline. At 2, 4, 6, and 9 weeks, five rats of each group were sacrificed after fasting for 18 h.

**Histopathology**

A small piece of liver tissue (about 5 mm × 5 mm × 5 mm) from each rat was fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.76 mM KH2PO4, pH 7.4). Five-micrometer paraffin sections were stained with Masson and James staining. The fibrosis score is defined using the following criteria: score 0, normal (no visible fibrosis); score 1, fibrosis present (collagen fiber present that extends from portal triad or central vein to peripheral region); score 2, mild fibrosis (mild collagen fiber present with extension without compartment formation); score 3, moderate fibrosis (moderate collagen fiber present with some pseudolobe formation); and score 4, severe fibrosis (severe collagen fiber present with thickening of the partial compartments and frequent pseudolobe formation).

**Preparation of liver PM for proteomic studies**

After sections were removed for histology, the remainder of each rat liver was used to purify PMs by sucrose density gradient ultracentrifugation as previously described [19].

**2-DE/MS technology**

2-DE was performed on an IPGphor isoelectric focusing system (Amersham Bioscience, Uppsala, Sweden) and Bio-Rad Protein II electrophoresis apparatus (Bio-Rad, Hercules, USA) as previously described [19,20]. Briefly, 1000 μg of protein was loaded on IPG gels (pH 3–10 NL) and focused with up to 52.1 Kvh. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (11.5%) were stained with Coomassie Blue G-250 and the 2-DE images were analyzed through ImageMaster 2D software (Amersham Bioscience). The differential protein spots were obtained by comparing the relative volume of each spot (dividing the volume of individual spot by the total volume of whole spots in the gel). If there was at least a 2-fold difference in the average volume of a spot in the gels from the alcohol-treated group in comparison with the average volume of that spot in the control gels, it was considered significant.

The differential proteins were then analyzed by Ultimate 3000 instruments ( Dionex, Germering, Germany) tandem nanoESI-MS-MS (mass spectrometry, Bruker Daltonics, Bremen, Germany) as previously described [19,21]. Briefly, the peptide mixture (5 μl) was directly subjected to a nanoLC-ESI-tandem MS system, including a PepMap™ C18 μ-pre-column (300 μm i.d. × 5 mm
The peptides were labeled with grade modified trypsin (1:10; Promega, Madison, USA). Proteins were digested overnight at 37°C for 60 min at a flow rate of 200 nl/min over 40 min. The continuous acetonitrile gradient consisted of 4–65% Solvent B (Solvent B, 80% ACN with 0.1% formic acid; Solvent A, 100% water with 0.1% formic acid). The eluted peptides from the reversed-phase nanocolumn were online injected onto a PicoTip emitter nanospray needle (New Objective, Woburn, USA) for real-time ionization and on a nanoESI-ion-trap mass spectrometer (Bruker Daltonics) for peptide fragmentation detection. Mascot 2.0.4 (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=MIS) was used for database search with the following parameters: enzyme, trypsin; allowance for up to one missed cleavage peptide; mass tolerance: 1.2 Da for MS and 0.6 Da for MS/MS; fixed modification parameter, carbamidomethyl (C); variable modification parameters, oxidation (at Met). Proteins were identified on the basis of peptides whose ions scores exceeded the threshold, \( P < 0.05 \), which indicated identification at the 95% confidence level for these matched peptides. The proteins identified by more than four peptides were accepted, without manual check. Proteins identified with less than three peptides were manually inspected. Each protein must have at least one peptide with four or more continue y- or b-series ions (e.g., y4, y5, y6, y7).

**Protein digest, iTRAQ labeling, and strong cation exchange fractionation**

iTRAQ labeling was done according to the kit protocol (ABI, Foster City, USA) and as previously reported [22–24]. Protein (100 \( \mu \)g) from the PM of alcohol-treated groups and normal controls were acetone-precipitated for 2 h at −20°C. After reduction and alkylation, protein solutions were digested overnight at 37°C with sequence grade modified trypsin (1:10; Promega, Madison, USA). The peptides were labeled with 30 \( \mu \)l iTRAQ™ Dissolution Buffer (ABI; 115 for alcohol-treated groups, 116 for the controls). The labeled peptides were pooled, desalted with Sep-Pak Cartridge (Waters, Milford, USA), and fractionated by strong cation exchange (SCX) chromatography on an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany) using a BioBasi SCX column (150 \( \times \) 2.1 mm, 5 \( \mu \)m; Thermo Fisher Scientific, Waltham, USA). Peptides were eluted with a linear gradient of 0–500 mM KCl (25% \( v/v \) acetonitrile, 10 mM KH₂PO₄, pH 2.8) for 60 min at a flow rate of 200 \( \mu \)l/min. Eight fractions were collected.

**LC-MS analysis**

Each SCX fraction was dried, dissolved in 0.1% formic acid, and analyzed on a Qstar Pulsar™ mass spectrometer (ABI/MDS Sciex, Concord, Canada). Peptides were separated on a reverse-phase column packed with ZORBAX 300SB-C18 enrichment column (5 \( \mu \)m, 300 Å, 0.5 \( \times \) 23 mm; Waters) and separated by a 75-\( \mu \)m i.d. PepMap™ RP column (Dionex) packed with 3-\( \mu \)m C18 beads with 100-Å pores. The buffers used were: Buffer A: 5% ACN, 95% water, 0.1% trifluoroacetic (TFA) and Buffer B: 95% ACN, 5% water, 0.1%TFA. The flow rate used for separation on the RP column was 400 nl/min with gradients 5–45% over 90 min. MS data were acquired automatically using Analyst QS 1.0 software Service Pack 8 (ABI/MDS Sciex). Analysis survey scans were acquired from 400 to 1800 m/z with up to six precursors selected for MS/MS from m/z 100–2000. The two most intense peaks over 30 counts, with a charge state 2–4, were selected for fragmentation. Curtain gas was set at 10, nitrogen was used as the collision gas, and the ionization tip voltage was 4000 V.

**Data analysis**

Ratios of the 115 (for alcohol groups) and 116 (for the controls) amu signature mass tags generated upon MS/MS fragmentation from the iTRAQ™-labeled tryptic peptides were calculated using Protein Pilot version 2.0.1 (ABI) in Analyst. The MS and MS/MS tolerances were set to 0.2 Da. The Swiss-Prot database was used for searching iTRAQ™-identified peptides. Methyl methanethiosulphonate modification of cysteines was used as a fixed modification, and one missed tryptic cleavage was allowed. All proteins identified had \( \geq 95\% \) confidence and the protein confidence threshold cutoff was set to 1.3 (unused) with at least more than one peptide above the 95% confidence level. The true value for the average ratio was expressed as an error factor (EF = \( 10^{0.05} \) confidence interval) and calculated as reported [20,25]. Protein quantification required an EF of less than 2 and a \( P\)-value < 0.05; only fold-changes \( >1.5 \) or \( <0.66 \) were considered significant. Data were exported and saved as excel files.

**Bioinformatics**

The theoretical isoelectric point (pI), molecular weight and exponentially modified protein abundance index (emPAI) were extracted from the Mascot web. The subcellular location and function of the identified proteins were elucidated by UniProt knowledgebase (Swiss-Prot/TrEMBL) and gene ontology (GO) database.

**Western blot analysis**

Fifty micrograms of total protein extracts was separated by electrophoresis in 11.5% SDS-PAGE gel and transferred to PVDF membrane (Millipore, Billerica, USA). Blots were incubated overnight at 4°C with the primary antibody, which was either mouse anti-annexin 2 (Santa Cruz Biotechnology, Santa Cruz, USA) at a 1:4000 dilution,
rabbit anti-keratin 8 (Abcam, Cambridge, USA) at a 1:4,000 dilution, rabbit anti-keratin 18 (Abcam) at a 1:5,000 dilution, or mouse anti-β-actin (Santa Cruz Biotechnology) at a 1:20,000 dilution. After three washes with TBST, blots were incubated for 1 h at 20°C with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies. The immune complexes were revealed by enhanced chemiluminescence and detected by X-rays.

**Immunohistochemistry**

Immunohistochemistry was performed as described previously. Sections (5 μm) of paraffin-embedded tissues were deparaffinized, hydrated, and washed three times in PBS. Subsequently, the slides were incubated overnight at 4°C with mouse anti-ANXA2 in a humidified chamber (dilution 1:100). The slides were washed in PBS for three times, incubated with horseradish peroxidase-conjugated anti-mouse antibody, and signals detected using a liquid 3,30-diaminobenzidine staining kit (Gene Tech Company, Shanghai, China), counterstained with hematoxylin–exon, dehydrated, mounted, and imaged digitally using an Olympus BX40 microscopy (Olympus, Tokyo, Japan) equipped with a color video camera.

**Statistical analysis**

The differential protein spots in gels were statistical analyzed by Student’s *t*-test. A *P* value < 0.05 was considered significant.

**Results**

**Histopathological findings**

As shown in Fig. 1, the liver fibrosis model was successfully established. At 2 weeks after injection, no substantial change was found in alcohol-treated group, liver fibrosis was diagnosed as S0 by Masson staining [Fig. 1(E, I)]. At 4 and 6 weeks, hepatic fibrosis was developed to S1 and S2–S3, respectively [Fig. 1(F, G)]. At 9 weeks, pseudolobules were developed, with the character of S3–S4 by Masson staining [Fig. 1(H, J)]. On the other hand, the liver of the control group showed no histopathological changes during the entire experiment. Furthermore, James staining was used to examine the histopathology of the liver fibrosis model as shown in Supplementary Fig. S1. The result showed no obvious difference was found during the growth from 2 to 9W in normal rats and the degree of liver fibrosis was increased gradually from S0 at 2W, S1 at 4W, S2–3 at 6W, and to S3–4 at 9W as indicated in green and highlighted by arrowhead.

**Purification of PM**

PMs were obtained by two-sequential sucrose density gradient ultracentrifugation and their purity was evaluated by western blot (Fig. 2). Fractions containing PM were identified by a PM marker enzyme, Na+/K+ ATPase and by decreased amounts of a mitochondrial marker, prohibitin. By comparing the area of signal bands analyzed by Quantity One 4.1.0 (Bio-Rad), we established that PM was enriched 10.1-fold in samples from the alcohol-treated group and 9.7-fold in the normal control, in comparison with the original homogenate. In the same samples, mitochondrial markers were decreased for 1.7- and 1.8 fold,
respectively. The total enrichment is about 17 fold (10.1 × 1.7 or 9.7 × 1.8) for both samples. The enrichment was consistent across different samples.

2-DE profiles of PM and identification of differential proteins

To obtain a detailed comparison of the differences in protein profiles, the PM proteins of both 2 week groups were extracted for 2-DE analysis. Analysis of the 2-DE images showed the average gel spots in the treated group and normal controls were 800 and 850 through 18-cm-2D gels (pI 3–10 NL) loaded with 1000-mg PM proteins (Fig. 3). On the basis of the relative volume (the volume of individual protein spots in comparison to the average spot volume in each gel), a total of 16 differential protein spots were detected, including 6 of increased volume and 10 of decreased volume. Figure 4(A) demonstrates a representative differential spot magnified in three gels containing samples from alcohol-treated animals vs. three gels from the controls. The corresponding image analysis result of this protein spot is shown in Fig. 4(B). The whole 2-DE gel images from triplicate samples were shown in Supplementary Fig. S2.

Among the 16 differential protein spots, 13 of them were identified by ESI-ion-trap MS, including 5 of increased volume (ratio_{Alcohol/Normal} ≥ 1.5, P ≤ 0.05) and 8 proteins that were decreased (ratio_{Alcohol/Normal} ≤ 0.66, P ≤ 0.05) (Fig. 3). They are involved in ten non-redundant proteins (Table 1). For these 13 proteins, 92% were identified by two or more peptides with 95% confidence. Only spot 9 (MRCKA_RAT) was identified by single peptide (Supplementary Table S1) with a mascot score of 68 (Table 1 and Supplementary Table S1), and MS/MS spectrum with continuous six y and four b ions (Supplementary Fig. S3). As shown in Table 1, except for spot 9, others were identified with more than 8% amino acids covered by MS. For example, in K8 (spot 6), 33% amino acids were detected by MS [Fig. 4(C)]. In order to make sure of the accuracy of protein identification by MS, the peptide spectra were checked manually. A representative peptide (LEVELGNMQGLVEDFK) from K8 was shown in Fig. 4(D). Eleven continuous y ions were detected by MS.

Identification of differentially expressed proteins by 2D-LC-MS

2D-LC-MS/MS strategy provides a powerful alternative to gel-based methods and shows superiority in analyzing hydrophobic proteins. A newly developed iTRAQ technique was used to compare differences in PM proteins between alcohol-treated rats and the controls. After LC-MS/MS analysis, 110 proteins were identified which had P-values > 95% confidence level (ProtScore > 1.3), and at least one peptide above 95% confidence level. Eighty-nine of them were quantified (Supplementary Table S2). Ninety-one percent (100 of 110) of proteins were identified by more than five peptides, 3.6% (4 out of 110) by four peptides, 1.8% (2 out of 110) by three peptides, 1.8% (2 out of 110) by two peptides, and only 1.8% (2 out of 110) by one peptide (Supplementary Table S3). Five differential proteins were detected (Table 2) according to the following criteria: (i) more than one peptide with 95% confidence; (ii) P < 0.05 and EF < 2 for the quality of quantification; (iii) iTRAQ regulation ratio of protein with ≥ 1.5-fold increase or < 0.66-fold decrease. Representative MS/MS spectra for three peptides identified from keratin 8 are shown in Fig. 5. Consistent changes were found in the three peptides [Fig. 5(A–C)]. Almost all y or b ions were detected in the sequence [Fig. 5(D)].
Through 2-DE/MS and iTRAQ-LC-MS strategies, 15 non-redundant differential proteins were identified, including 9 of decreased abundance and 6 of increased abundance. Surprisingly, only one protein was simultaneously identified by both methods. Ten distinct proteins were identified through 2-DE/MS technology (Table 1), whereas five distinct proteins were identified through iTRAQ (Table 2). The data presented herein strongly support the complementary nature of 2-DE/MS and iTRAQ labeling methods.

**Bioinformatic analysis of differential proteins**

Molecular functions and possible subcellular distribution of the 15 differential proteins were further explored using UniProt knowledgebase (Swiss-Prot/TrEMBL) and GO database. Eighty-percent (12 of 15) differential proteins were located in PM, including proteins annotated as membrane, basement membrane, intermediate filament, keratin filament, cell–cell junction, extrinsic to external side of PM, axon, as well as PM. There are also proteins located in other subcellular organelles such as one in nucleus, one in cytoplasm, and one in endoplasmic reticulum (ER) (Tables 1 and 2). Furthermore, we examined the protein function annotated by GO database, and found proteins involved in the following biological processes to be among the most frequently changed: binding (49%), enzyme activities (31%) and cell structure (8.0%) (Tables 1 and 2).

**Western blot analysis**

The dynamic changes of those differentially expressed proteins: ANXA2, K8 (Keratin, type II cytoskeletal 8) and K18 (keratin, type 1 cytoskeletal 18) in the rat model PM at 2, 6, and 9 weeks were validated by western blot analyses. β-Actin was used as internal control. ANXA2 was verified to be significantly increased, while K8 and K18 were decreased in the liver PM of alcohol-treated rats compared with the controls (Fig. 6).

**Immunohistochemistry**

Immunohistochemical studies were performed in the rat model biopsy at 2, 6, and 9 weeks using ANXA2 antibodies. The immunohistochemical analysis revealed a good correlation between the antibody staining and the proteome expression profiles obtained by 2-DE.

At 2 weeks, very little positive signal was detected in rat liver hepatocytes and fibroblasts [Fig. 7(D)]. At 6 weeks, positive signal was found in hepatocytes and even stronger in fibroblast [Fig. 7(E)]. At 9 weeks, stronger ANXA2 staining was detected [Fig. 7(F)]. In contrast, only little positive signal can be detected in hepatocytes from 2 to 9 weeks in the controls [Fig. 7(A–C)].

**Figure 4** Representative image analysis and MS identification of 2-DE/MS-based strategy (A) Magnified regions of 2D images showing the six spots, which had a decreased volume in samples from ethanol-treated rats (right) when compared with those from controls (left). (B) Image analysis result of the six spots. (C) Sequence coverage of keratin 8 identified by MS. Amino acids detected by MS are shown in bold and highlighted by underline. (D) MS/MS spectrum of peptide (LEVELGNQGLVEDFK) from keratin 8.
Liver diseases afflict more than 10% of the world’s population. Chronic alcohol overconsumption is one of the main causes for liver fibrosis, cirrhosis, and liver cancer. However, strategies for the diagnosis, staging, and treatment of these liver diseases remain limited. Fibrosis and its consequence cirrhosis, represent long-term processes, which are associated with major modifications of extracellular matrix (ECM) proteins. Before fibrosis and cirrhosis can be easily observed at the histological level, many changes in the protein expression profile of hepatic fibroblasts are thought to occur. Proteomics, a new frontier in functional genomics, with many benefits, has been widely used to search for new biomarkers [26–28] and to understand the mechanisms of fibrosis [29]. In our previous study, we identified ANXA2 in immune liver fibrosis through PM proteome analysis [19]. However, in all of these studies [19,26–29], liver fibrosis had already been developed. The knowledge of early protein changes before liver fibrosis is very limited. In the present study, we used proteomics to uncover alterations in liver protein profiles

![Table 1 Differential proteins identified by 2-DE/MS technology](#)

<table>
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<tr>
<th>Spot number</th>
<th>Accession number</th>
<th>Protein description</th>
<th>Regulation (ratio)</th>
<th>Score</th>
<th>pI</th>
<th>Molecular mass (kDa)</th>
<th>Coverage (%)</th>
<th>Functiona</th>
<th>Locationb</th>
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<td>TPM3_RAT</td>
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*aBinding including: DNA binding, cell surface binding, copper ion binding, drug binding, fatty acid binding, protein binding, pyridoxal phosphate binding, toxin binding and zinc ion binding.

*bPM including membrane, basement membrane, intermediate filament, keratin filament, cell–cell junction, extrinsic to external side of PM, axon, as well as PM.

*cProteins expressed only in control samples.

**Discussion**

LIVER DISEASES AFFLICT MORE THAN 10% OF THE WORLD’S POPULATION. CHRONIC ALCOHOL OVERCONSUMPTION IS ONE OF THE MAIN CAUSES FOR LIVER FIBROSIS, CIRRHOSIS, AND LIVER CANCER. HOWEVER, STRATEGIES FOR THE DIAGNOSIS, STAGING, AND TREATMENT OF THESE LIVER DISEASES REMAIN LIMITED. FIBROSIS AND ITS CONSEQUENCE CIRRHOSIS, REPRESENT LONG-TERM PROCESSES, WHICH ARE ASSOCIATED WITH MAJOR MODIFICATIONS OF EXTRACELLULAR MATRIX (ECM) PROTEINS. BEFORE FIBROSIS AND CIRRHOSIS CAN BE EASILY OBSERVED AT THE HISTOLOGICAL LEVEL, MANY CHANGES IN THE PROTEIN EXPRESSION PROFILE OF HEPATIC FIBROBLASTS ARE THOUGHT TO OCCUR. PROTEOMICS, A NEW FRONTIER IN FUNCTIONAL GENOMICS, WITH MANY BENEFITS, HAS BEEN WIDELY USED TO SEARCH FOR NEW BIOMARKERS [26–28] AND TO UNDERSTAND THE MECHANISMS OF FIBROSIS [29]. IN OUR PREVIOUS STUDY, WE IDENTIFIED ANXA2 IN IMMUNE LIVER FIBROSIS THROUGH PM PROTEOME ANALYSIS [19]. HOWEVER, IN ALL OF THESE STUDIES [19,26–29], LIVER FIBROSIS HAD ALREADY BEEN DEVELOPED. THE KNOWLEDGE OF EARLY PROTEIN CHANGES BEFORE LIVER FIBROSIS IS VERY LIMITED. IN THE PRESENT STUDY, WE USED PROTEOMICS TO UNCOVER ALTERATIONS IN LIVER PROTEIN PROFILES...
before fibrosis was induced by alcohol. To our knowledge, this is the first demonstration of changes in the PM proteome prior to the development of liver fibrosis in the rat model.

To identify more membrane proteins, two technologies, 2-DE/MS and iTRAQ, were used. Although 2-DE/MS technology has some limitation for membrane proteome analysis, it is still widely used for 90% of the predicted

Table 2 Differential proteins identified by iTRAQ technology

<table>
<thead>
<tr>
<th>Number</th>
<th>Unused Coverage (95%)</th>
<th>Accession number</th>
<th>Name</th>
<th>Peptides (95%)</th>
<th>Regulation (ratio)</th>
<th>PVal</th>
<th>EF</th>
<th>Function</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>32.55</td>
<td>9.94</td>
<td>MYH9_RAT</td>
<td>Myosin-9</td>
<td>17</td>
<td>0.28</td>
<td>4.61E-06</td>
<td>1.94</td>
<td>Binding; motor; enzyme</td>
</tr>
<tr>
<td>17</td>
<td>7.93</td>
<td>6.74</td>
<td>ALBU_RAT</td>
<td>Serum albumin</td>
<td>3</td>
<td>0.47</td>
<td>0.016</td>
<td>1.28</td>
<td>Binding</td>
</tr>
<tr>
<td>8</td>
<td>19.25</td>
<td>5.99</td>
<td>SPTA2_RAT</td>
<td>Spectrin alpha chain, brain</td>
<td>9</td>
<td>0.57</td>
<td>0.042</td>
<td>1.53</td>
<td>Binding</td>
</tr>
<tr>
<td>2</td>
<td>36.85</td>
<td>36.02</td>
<td>K2C8_RAT</td>
<td>Keratin, type II cytoskeletal 8</td>
<td>20</td>
<td>0.66</td>
<td>0.006</td>
<td>1.11</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>8.72</td>
<td>34.4</td>
<td>H2B1_RAT</td>
<td>Histone H2B type 1</td>
<td>5</td>
<td>2.86</td>
<td>0.003</td>
<td>1.49</td>
<td>Binding</td>
</tr>
</tbody>
</table>

The peptides from alcohol-treated or control groups were labeled with 115- or 116-reporter isobaric reagent, respectively. Coverage (95%), sequence coverage at the 95% confidence level; PVal, P-value, a P-value < 0.05 was significant for protein quantification; EF, error factor, if the error factor is >2, the average ratio is far from the actual value.

Figure 5 Representative MS result of iTRAQ-LC-MS strategy  Examples of MS and MS/MS spectra of a 1.5-fold decreased protein (keratin 8), illustrating the degree of consistency in relative quantitative measurement. Samples from ethanol-treated rats and the controls were labeled with 115- and 116-reporter isobaric reagent, respectively. Three quantified tryptic peptides (from a total of 61 non-redundant peptides) were shown in A, B and C. The MS spectrum of a peptide—FASFIDK with m/z of 558.3165 (z = 2) was shown in (A), YEELQTLAGK, m/z = 720.4106 and z = 2 in (B), LEGLTDEINFLR, m/z = 782.4341, and z = 2 in (C). The MS/MS spectrum of the peptide (LEGLTDEINFLR), related b and y ions are shown in D. The signal intensity for reporters and background were shown in crosswise lines. The difference between reporter and background is the actual value of the 115- or 116 reporter.
yeast membrane proteins with a grand average of hydropathy (GRAVY) value <0.4 [9], which falls into the solubility range commonly detected on 2D gels. Our previous study [30] also showed that PM proteins can be better separated by 2-DE through an optimized extraction method. So in this work, 2-DE methodology was used to analyze the liver PM proteome, and 13 protein spots with a greater than 2-fold difference were identified.

iTRAQ is a high-content proteomic technique for substrate degradomics, and can be used to label four or eight samples simultaneously [31–33]. Through iTRAQ technology, five differential proteins were identified; fewer than that were identified through 2-DE/MS. Only one protein was identified by two technologies. These might be due to the reason that only eight fractions were collected and analyzed by LC-MS.

For the identified proteins, even with twice sucrose density gradient ultracentrifugation to purify PM and obtain the membrane with more than 9-fold enrichment of PM markers and nearly 2-fold depletion of specific markers of mitochondria compared with homogenate, there were still some other organelle’s proteins identified such as calreticulin, histone, and serum albumin. These might due to two reasons: (1) the multiple locations of proteins [34,35], (2) the contamination of other organelles such as mitochondrial membrane, cytosol and blood. There is no technology that can remove other organisms from PM completely so far. So our method can offer some help to lower abundant PM protein research, although PM is not enriched very well in this work.

According to GO database, the differential proteins mainly were those with binding activities such as DNA binding; cell surface binding and actin binding (49%), and enzyme activities such as protein disulfide-isomerase and serine/threonine-protein kinase MRCK alpha (31%). These results indicated that the cell signal transport, biochemistry reaction and cell structure were mainly regulated during liver fibrogenesis. Of course, it cannot be excluded that the proteins with binding and enzyme activities are more than others in cell proteome.

In this work, three differential proteins (ANXA2, K8 and K18) were verified by western blotting. According to previous reports [19,36,37], alcohol can directly up-regulate the expression of ANXA2 [36] in cells. ANXA2 was reported to be related to liver fibrosis caused by alcohol [37] and by serum [19]. In this work, we also detected an increase in the amount of PM-associated ANXA2 after alcohol treatment for 2 weeks, although liver fibrosis was not yet developed at that stage. Our data indicate that ANXA2 is an early effector molecule during the progression of liver fibrosis. This study is a complementary to the previous reports that alcohol directly affects ANXA2 [36] and ANXA2 is a protein related to liver fibrosis [19,37]. K8 and K18 are cytoprotective stress proteins that play a central role in guarding hepatocytes from apoptosis [38,39]. Keratin can modulate disease progression upon

![Figure 6 Western blot analysis of the selected proteins](image)

Figure 6 Western blot analysis of the selected proteins Fifty micrograms of PM protein extracts at 2 weeks was separated on 11.5% separation gels. β-Actin was used as a loading control. N2, N6, and N9 represent liver PM-enriched samples from 2-, 6- and 9-week normal controls, respectively. A2, A6, and A9 represent liver PM-enriched samples from 2-, 6- and 9-week alcohol-treated rat models, respectively.

![Figure 7 Immunohistochemistry analysis of the differential protein, annexin A2](image)

Figure 7 Immunohistochemistry analysis of the differential protein, annexin A2 Rats were treated with saline for 2, 6, and 9 weeks (A, B, and C, 40×), and with alcohol for 2, 6, and 9 weeks (D, E, and F, 40×), respectively. The immunohistochemical analysis revealed a good correlation between the antibody staining and the proteome expression profiles obtained by 2DE.
mutation, and serve as a marker of epithelial cell death [38]. Keratin filament collapse is a major risk for acute liver injury [40]. K8/K18 mutations are present in a German population and collectively associate with progression of fibrosis in chronic hepatitis C (CHC) infection [41]. Keratin 8/18 hyperphosphorylation is a marker of progression of human liver disease. Site-specific keratin phosphorylation in liver disease is a progression marker when increased and a likely regression marker when decreased [42]. Furthermore, according to Cohen et al. [43], proteins can rapidly translocate under drug’s stimulation. So the down-regulation of K8 and K18 found in this work might be due to the following reasons: (i) K8 and K18 genes mutations; (ii) phosphorylation; (iii) protein translocation. In the future, it will be interesting to study K8 and K18 expression from these three points. In all, our study might offer two complements to the knowledge of K8 and K18: (i) K8 and K18 are related to the development of liver fibrosis; (ii) during liver fibrosis development, K8 and K18 were down-regulated in PM.

In summary, we applied a subcellular proteomic approach to identify proteins as potential candidate biomarkers for preventing liver fibrosis. In addition to ANXA2, which was a common protein of hepatic fibrogenesis, novel candidate biomarkers such as K8 and K18 were identified, and subsequently confirmed by western blotting. We also identified other proteins such as tropomyosin alpha-3 chain, calreticulin, and annexin A3. These results might be helpful for understanding the mechanism of alcohol-induced liver fibrosis and looking for drug targets to prevent the development of alcohol-induced liver disease. Further studies on this field will be interesting, such as post-translation modification of K8 and K18, dynamic protein translocation between PM, and other organelles.

**Supplementary data**

Supplementary data is available at *ABBS* online.

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