Original Article
Liver protein spectrum in a rat liver injury model

Huan Wang1*, Shan Tong2*, Hongmei Han3, Tuul Khalzaabaast4, Bagenna Bao1

1School of Mongolian Medicine and Pharmaceutical Sciences, 2College of Mongolian Medicine, Inner Mongolia University for The Nationalities, Tongliao, Inner Mongolia, P. R. China; 3Mongolian Medicine Surgery, 4Mongolian Medicine Digestive Internal Medicine, Affiliated Hospital of Inner Mongolia University for The Nationalities, Tongliao, Inner Mongolia, P. R. China. *Co-first authors.

Received May 15, 2017; Accepted December 13, 2017; Epub July 15, 2018; Published July 30, 2018

Abstract: This study aimed to identify the potential key proteins associated with liver injury based on the established rat model. The rat liver injury model was established using the method of pyloric ligation and then was confirmed by determination of serum enzymes and bilirubins as well as liver glutathione peroxidase and liver index. The protein spectrum of liver samples was analyzed using high performance liquid chromatography-mass spectrometry system. Subsequently, the differentially expressed proteins (DEPs) between the model rats and controls were identified, and their potential functions and interactions were further analyzed. The rat liver injury model was successfully established. According to the analysis of liver protein spectrum, a total of 147 DEPs were identified, including 80 upregulated and 67 downregulated DEPs in the model group. In the PPI network, upregulated Stat3 which had the highest degree interacted with multiple upregulated proteins, such as Junb, Fgg, and Hmox1, as well as downregulated proteins like Serpina3c and Tgfb1i1. Furthermore, Stat3 and Hmox1 were predicted to be associated with immunity. The DEPs may play crucial roles in the liver injury. These results are expected to contribute to the diagnosis and therapy of liver injury.

Keywords: Differentially expressed protein, liver injury, model, network, protein spectrum

Introduction

Idiosyncratic drug-induced liver injury (DILI) is a common event because of the expanding use of prescription drugs, accounting for about 13% of acute liver failure cases in the United States [1]. It is the most frequent adverse drug reaction (ADR) that leads to termination of development programs for new drugs, failure of approval by supervision agencies, even withdrawal of licensed drugs [2]. Despite the great effect of DILI on both development of important new medications and patient health, the risk factors and mechanisms of idiosyncratic ADRs that lead to liver injury are still little understood.

In the past few years, a set of proteins and biological functions have been found to be associated with DILI. For example, a mass spectrometry (MS)-based quantitative proteomic approach has identified 92 differentially expressed priority 1 proteins between the DILI patients and controls [3]. This study has also discovered that apolipoprotein E expression is able to differentiate DILI patients from the controls [3]. Deficiency of transmembrane thioredoxin-related protein can cause increased sensitivity to the toxicants and developed severe liver damage [4]. Furthermore, activator protein 1 (AP-1) proteins, such as Jun and Fos, have also been reported to be implicated in the development of liver injury [5]. Additionally, a recent study has reported that the phosphorylation of CCAAT/Enhancer Binding Protein-β (C/EBPβ)-Thr217 is essential for the activation of the inflammatory in liver macrophages and liver injury [6]. However, numerous proteins and protein-protein interactions (PPIs) that are related to liver injury have still not been detected.

In this study, pyloric ligation was used to induce rat liver injury, and high performance liquid chromatography-MS (HPLC-MS) system was utilized to scan the liver protein spectrum in rat liver injury models and controls. Expression difference of proteins was analyzed, and the potential functions and interactions of the differentially expressed proteins (DEPs) were further investigated. These results were expected to be helpful for a better understanding of the pathogenesis of liver injury, and provide potential biomarkers for the diagnosis and therapy of liver injury.
Protein spectrum in liver injured rat

Materials and methods

Animals and grouping

A total of 20 specific-pathogen-free Wistar male rats (180-220 g) were purchased from Changchun Yisi experimental animal technology co., LTD (certification number: SCXK (JL)-2011-0004, Changchun, China). According to the random number table, all of rats were divided into five groups randomly: the model group, and control group. Each group included 10 rats. All of rats were fed under pathogen-free conditions and underwent a reversed 12:12 h light/dark cycle. The room temperature was kept constant at 22-25°C, and the relative humidity at 50%. After two days of adaptation, all of rats underwent fasting but not water deprivation for 24 h. Subsequently, the animals were narcotized by 10% chloral hydrate. For animals in the model group, they received pyloric ligation, and then underwent fasting but not water deprivation for 18 h. These animals were fed separately. Animals in the control group did not undergo any surgery. Afterwards, they were narcotized by 10% chloral hydrate, and blood was sampled from abdominal aorta. The liver was also sampled, washed and weighed. Part of the liver sample was immediately quick-frozen in liquid nitrogen and then stored at -80°C.

Determination of blood and liver indexes

Serum aminotransferase levels [aspartate aminotransferase (AST) and alanine aminotransferase (ALT)], serum direct bilirubin (BILD2), and serum total bilirubin (BILT3) were measured using the Roche test kits. The levels of serum alkaline phosphatase (ALP) were determined using an alkaline phosphatase kit (Roche Molecular Biochemicals). Furthermore, the activity of glutathione peroxidase (GSH-PX) in the liver was determined using the test kit obtained from the Nanjing Jiancheng biological engineering center (Nanjing, China). Additionally, following a liver index was calculated as liver mass/rat mass × 100%.

Tandem mass tag (TMT)-labeled protein spectrum

Proteins in the liver samples were extracted using the radio immunoprecipitation assay buffer method. After the quantitative determination by BCA Protein Assay Kit (Pierce), 100 µg protein in each sample was used for the TMT labeling, which was conducted according to the instructions of the TMT isobaric Mass Tagging Kits and Reagents (Pierce). Subsequently, the TMT-labeled protein samples underwent vacuum concentration, and then were dissolved. The labeled protein solution (pH 10) was centrifuged and the supernatant was graded in the C18 column using a gradient elution pattern. The 0.1% formic acid mixed with 99.9% H2O was used as A phase in LC, and 99.9% acetonitrile mixed with 0.1% formic acid was used as B phase in LC. Flow rate was set as 300 nL/min.

After the gradient elution, samples were input into the Q Exactive high performance liquid chromatography (LC) system (Thermo Fisher Scientific, Hudson, NH, USA) equipped with NCS3500 MS system (scan range: m/z 350-1600, Dionex, Sunnyvale, CA, USA). The 0.1% formic acid mixed with 99.9% H2O was used as A phase in LC, and 99.9% acetonitrile mixed with 0.1% formic acid was used as B phase in LC. Flow rate was set as 300 nL/min.

Data preprocessing and identification of DEPs

The proteome database (human-refseq-20140-303-71465s.fasta) downloaded from National
Protein spectrum in liver injured rat

Center of Biotechnology Information (NCBI) was used for the analysis of the generated protein spectrum data by the Proteome Discoverer software. The relatively credible data of polypeptides and proteins were retained. Afterwards, the DEPs between the model and control groups were identified based on the expression data of polypeptides and proteins. The cut-off criteria were set as false discovery rate (FDR) < 0.05 and fold change (FC) > 1.5.

Functional enrichment analyses of DEPs

Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were carried out using KOBAS (KEGG Orthology Based Annotation System, http://web.ttsnetwork.net/KS/pages/in dex.jsp) [7]. P-value < 0.05 was set as the threshold.

Construction of protein-protein interaction (PPI) network

For all of the identified DEPs, PPI network was constructed with information from a well-known online server, Search Tool for the Retrieval of Interacting Genes/Proteins version 10 (STRING v10) [8]. Only the PPIs with confidence score > 0.4 were defined as significant PPIs, which were utilized to construct the PPI network. Network was visualized using software Cytoscape version 2.8 [9]. In the network, node represents a protein; line represents the interaction between two proteins; and the degree of a node is equal to the number of nodes interacting with this node.

Statistical analysis

Data were presented as mean ± standard deviation from three separate experiments performed in duplicate. Statistical analysis was performed using SPSS 19.0 software (SPSS, Chicago, USA). The unpaired Student t-test was used to compare group means. Differences were considered significant if P < 0.05.

Results

Effect of pyloric ligation on the expression of serum enzymes and bilirubins

The expression levels of serum AST, ALT, and ALP were significantly increased in the model group than in the control group (P < 0.05 or 0.01). Furthermore, the levels of BILD2 and BILT3 were also significantly higher in the model group than in the control group (P < 0.01) (Table 1).

Effect of pyloric ligation on the content of liver GSH-PX and liver index

The GSH-PX in liver was significantly lower in the model rats than in the controls (P < 0.05, 210.76 U·L⁻¹ vs. 259.12 U·L⁻¹). By contrast, the liver index in the in the model rats was significantly higher than in the controls (P < 0.05, 2.88% vs. 2.67%) (Table 2).

Identified DEPs

Based on the protein spectrum data, using the criterion of 1% FDR, 6095 kinds of proteins and
47,538 kinds of polypeptides were identified and quantified. Following the criteria of FDR < 0.05 and FC > 1.5, a total of 147 DEPs were identified, including 80 upregulated and 67 downregulated.
Protein spectrum in liver injured rat

7196


downregulated DEPs. The clustering heat map revealed that these DEPs were able to distinguish the model samples from the control samples (Figure 1).

Potential functions of the DEPs

To further reveal the potential biological functions of the identified DEPs, the GO and KEGG pathway enrichment analyses were performed. A total of 18 significant GO terms in biological process (BP) and 3 terms in cellular component (CC) were enriched by the DEPs. For example, Hmox1 and Fgg were involved in GO terms like “defense response” and “inflammatory response”; Junb was related to “response to cytokine” (Table 3).

Analysis of the PPI network

To further investigate the interactions of the DEPs, a PPI network was constructed. The PPI network consisted of 63 proteins and 73 PPI pairs. In this network, upregulated Stat3 had the highest degree, and it interacted with 10 proteins, such as upregulated Junb and Hmox1, as well as downregulated Serpina3c and Tgfb1i1 (Figure 2).

Furthermore, the potential functions of proteins in the PPI network were explored. A series of significant GO terms were enriched by the proteins in the network. For instance, Stat3 and Hmox1 were enriched in the GO terms about immunity, such as “defense response”, “inflammatory response”, and “response to cytokine stimulus” (Table 4). Meanwhile, 9 significant KEGG pathways were enriched by the proteins, such as “complement and coagulation cascades” (e.g. Fgg, Fga, Fgb), and “steroid hormone biosynthesis” (e.g. Ugt1a2) (Table 5).

Discussion

In the present study, the rat liver injury models were established by pyloric ligation. The liver index and the expression levels of serum AST, ALT and ALP, as well as BILD2 and BILT3, were significantly higher in the model group than in the control group. By contrast, liver GSH-PX was significantly lower in the model rats than in the controls. These results indicated that the rat liver injury models were successfully established. Furthermore, the liver protein spectrum was analyzed using HPLC-MS. A total of 147 DEPs were identified, including 80 upregulated and 67 downregulated DEPs. In the PPI network, upregulated Stat3 had the highest degree, and it interacted with upregulated proteins such as Junb, Fgg, and Hmox1, as well as downregulated Serpina3c and Tgfb1i1.

Stat3 encodes signal transducer and activator of transcription 3, which is activated by tyrosine phosphorylation during the response to interleukin-6 (IL-6) and epidermal growth factor [10]. In this study, Stat3 was predicted to be associated with immunity. In the liver acute phase response, STAT3 plays a key role along with IL-6 [11]. Multiple previous studies have found that STAT3 is associated with liver injury. For instance, Stat3 signaling activation cross-linking transforming growth factor-β1 in hepatic stellate cell contributes to the exacerbation of liver injury and fibrosis [12]. IL-6/STAT3 signal-
### Table 4. The top 10 enriched GO terms in each category by the proteins in the PPI network

<table>
<thead>
<tr>
<th>Category</th>
<th>Term</th>
<th>Gene count</th>
<th>P-value</th>
<th>Gene symbols of proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP</td>
<td>GO: 0006952—defense response</td>
<td>13</td>
<td>7.00E-08</td>
<td>Kng1, Lyz2, II1R1, S100A8, Camp, Kggl1, II1Rn, Stat3, Serpina3N, Fgg, Hmox1, Np4, Fn1</td>
</tr>
<tr>
<td>BP</td>
<td>GO: 0009611—response to wounding</td>
<td>12</td>
<td>1.16E-06</td>
<td>Kng1, Pcsk1, Fgg, Serpina3N, Fga, S100A8, Fgb, Hmox1, II1Rn, Kggl1, Stat3, Fn1</td>
</tr>
<tr>
<td>BP</td>
<td>GO: 0010036—response to inorganic substance</td>
<td>10</td>
<td>1.52E-06</td>
<td>Pcsk1, Fgg, Fga, M1A1, Fgb, Hmox1, M2A2, Anxa1, Abat, Sod3</td>
</tr>
<tr>
<td>BP</td>
<td>GO: 0006954—inflammatory response</td>
<td>9</td>
<td>3.10E-06</td>
<td>Kggl1, Serpina3N, S100A8, Hmox1, II1Rn, Kggl1, Stat3, Fn1</td>
</tr>
<tr>
<td>BP</td>
<td>GO: 0034097—response to cytokine stimulus</td>
<td>7</td>
<td>7.08E-06</td>
<td>Pcsk1, II1R1, Serpina3N, Anxa1, Litr, Junb, Stat3</td>
</tr>
<tr>
<td>BP</td>
<td>GO: 0010038—response to metal ion</td>
<td>8</td>
<td>8.60E-06</td>
<td>Pcsk1, Fgg, Fga, M1A1, Fgb, M2A2, Abat, Sod3</td>
</tr>
<tr>
<td>BP</td>
<td>GO: 0006953—acute-phase response</td>
<td>5</td>
<td>1.05E-05</td>
<td>Kggl1, II1Rn, Kggl1, Stat3, Fn1</td>
</tr>
<tr>
<td>BP</td>
<td>GO: 0014070—response to organic cyclic substance</td>
<td>8</td>
<td>2.69E-05</td>
<td>Pcsk1, Hmox1, II1Rn, Adlh2, Anxa1, Abat, Junb, Stat3</td>
</tr>
<tr>
<td>BP</td>
<td>GO: 0048545—response to steroid hormone stimulus</td>
<td>8</td>
<td>2.03E-04</td>
<td>Pcsk1, Ugt1A2, Hmox1, II1Rn, Adlh2, Anxa1, Junb, Stat3</td>
</tr>
<tr>
<td>BP</td>
<td>GO: 0006917—response to bacterium</td>
<td>7</td>
<td>2.52E-04</td>
<td>Kggl1, Lyz2, Camp, II1Rn, Np4, Adlh2, Mgst1</td>
</tr>
<tr>
<td>CC</td>
<td>GO: 0005615—extracellular space</td>
<td>14</td>
<td>6.10E-08</td>
<td>Kggl1, II1Rn, Kggl1, Anxa1, Igfls, Hgf, Sod3, Pcsk1, Azgol, Fgg, Fga, Fgb, Np4, Fn1</td>
</tr>
<tr>
<td>CC</td>
<td>GO: 0005577—fibrinogen complex</td>
<td>4</td>
<td>2.71E-07</td>
<td>Fgg, Fga, Fgb, Fn1</td>
</tr>
<tr>
<td>CC</td>
<td>GO: 0044421—extracellular region part</td>
<td>14</td>
<td>3.08E-06</td>
<td>Kggl1, II1Rn, Kggl1, Anxa1, Igfls, Hgf, Sod3, Pcsk1, Azgol, Fgg, Fga, Fgb, Np4, Fn1</td>
</tr>
<tr>
<td>CC</td>
<td>GO: 0030141—secretory granule</td>
<td>8</td>
<td>2.06E-05</td>
<td>Pcsk1, Fgg, Lyz2, Fga, Fgb, Camp, Hgf, Fn1</td>
</tr>
<tr>
<td>CC</td>
<td>GO: 0031093—platelet alpha granule lumen</td>
<td>5</td>
<td>2.17E-05</td>
<td>Fgg, Fga, Fgb, Hgf, Fn1</td>
</tr>
<tr>
<td>CC</td>
<td>GO: 0060205—cytoplasmic membrane-bounded vesicle lumen</td>
<td>5</td>
<td>2.91E-05</td>
<td>Fgg, Fga, Fgb, Hgf, Fn1</td>
</tr>
<tr>
<td>CC</td>
<td>GO: 0005576—extracellular region</td>
<td>17</td>
<td>3.22E-05</td>
<td>Kggl1, Camp, II1Rn, Kggl1, Anxa1, Igfls, Hgf, Sod3, Azgol, Pcsk1, Serpina3N, Fgg, Fga, Hpx, Fgb, Np4, Fn1</td>
</tr>
<tr>
<td>CC</td>
<td>GO: 0031983—vesicle lumen</td>
<td>5</td>
<td>4.52E-05</td>
<td>Fgg, Fga, Fgb, Hgf, Fn1</td>
</tr>
<tr>
<td>CC</td>
<td>GO: 0031091—platelet alpha granule lumen</td>
<td>5</td>
<td>6.69E-05</td>
<td>Fgg, Fga, Fgb, Hgf, Fn1</td>
</tr>
<tr>
<td>CC</td>
<td>GO: 0031982—vesicle</td>
<td>11</td>
<td>2.18E-04</td>
<td>Pcsk1, Fgg, Lyz2, Fga, Ngo, Fgb, Camp, II1Rn, Np11, Hgf, Fn1</td>
</tr>
<tr>
<td>MF</td>
<td>GO: 0030674—protein binding, bridging</td>
<td>4</td>
<td>5.91E-04</td>
<td>Fgg, Fga, Fgb, Anxa1</td>
</tr>
<tr>
<td>MF</td>
<td>GO: 0043499—eukaryotic cell surface binding</td>
<td>3</td>
<td>1.74E-03</td>
<td>Fgg, Fga, Fgb</td>
</tr>
<tr>
<td>MF</td>
<td>GO: 0043498—cell surface binding</td>
<td>3</td>
<td>5.04E-03</td>
<td>Fgg, Fga, Fgb</td>
</tr>
<tr>
<td>MF</td>
<td>GO: 0008236—serine-type peptidase activity</td>
<td>5</td>
<td>6.54E-03</td>
<td>Pcsk1, Sec11C, Prepi, Hgf, Ctsg</td>
</tr>
<tr>
<td>MF</td>
<td>GO: 0017171—serine hydrolase activity</td>
<td>5</td>
<td>6.65E-03</td>
<td>Pcsk1, Sec11C, Prepi, Hgf, Ctsg</td>
</tr>
<tr>
<td>MF</td>
<td>GO: 0005506—iron ion binding</td>
<td>5</td>
<td>2.12E-02</td>
<td>Steap4, Cyp17A1, Hpx, Hmox1, Cyp2C22</td>
</tr>
<tr>
<td>MF</td>
<td>GO: 0005507—copper ion binding</td>
<td>3</td>
<td>2.24E-02</td>
<td>Steap4, Mt1A, Sod3</td>
</tr>
<tr>
<td>MF</td>
<td>GO: 0004252—serine-type endopeptidase activity</td>
<td>4</td>
<td>2.55E-02</td>
<td>Pcsk1, Prepi, Hgf, Ctsg</td>
</tr>
<tr>
<td>MF</td>
<td>GO: 0019838—growth factor binding</td>
<td>3</td>
<td>4.23E-02</td>
<td>II1R1, Litr, Igfls</td>
</tr>
<tr>
<td>MF</td>
<td>GO: 0046872—metal ion binding</td>
<td>17</td>
<td>4.25E-02</td>
<td>Steap4, S100A8, S100A9, Anxa1, Adlh2, Stat3, Sod3, Pcsk1, Cyp17A1, Fgg, Mt1A, Hpx, Hmox1, M2A2, Fgfl1, Fn1, Cyp2C22</td>
</tr>
</tbody>
</table>

GO, Gene Ontology; BP, biological process; CC, cellular component; MF, molecular function; PPI, protein-protein interaction.

Protein spectrum in liver injured rat

In this study, upregulated proteins such as Junb, Fgg and Hmox1 were predicted to interact with Stat3. Junb (Jun B Proto-Oncogene) is involved in multiple cell processes, such as cell proliferation, differentiation and invasion [16, 17]. In the liver injury mice, Junb expression is altered during the liver regeneration [18]. A previous study has discovered that JUNB is specifically expressed in human and murine immune cells during acute liver injury, and it mediates the expression of interferon-γ [19]. Hmox1 interacted with both Stat3 and Junb in the cur-
rent study. During liver regeneration after liver injury in mice, Hmox1 is highly expressed [20, 21]. Fgg encodes the gamma component of fibrinogen, which has a major function in hemostasis as one of the primary components of blood clots [22]. Fgg has been found to be highly expressed in liver-injured mice, compared with the controls [15]. These results suggest that Junb, Fgg, and Hmox1 may also play critical roles in the liver injury likely via the interaction with Stat3.

Furthermore, several downregulated proteins were predicted to interact with Stat3, such as Serpina3c and Tgfb1i1. Serpina3c protein is a serine protease inhibitor [23]. Previous studies have reported that Serpina3c takes part in the immune system [24, 25]. Tgfb1i1 (transforming growth factor beta 1 induced transcript 1), also known as HIC5, is a coactivator of the androgen receptor [26]. A recent study has demonstrated that deficiency of Hic-5 weakens the activation of mice hepatic stellate cells and liver fibrosis via the upregulation of Smad7 [27]. There is no other study to support the associations of Serpina3c and Tgfb1i1 with liver injury. We speculated that these two proteins might participate in liver injury via immunity or the interaction with Stat3.

Despite the aforementioned results, there were several limitations in this study. The predictions needed to be confirmed by experiments. In our further study, the expressions of the proteins (e.g. Stat3, Junb, Fgg, Hmox1, Serpina3c and Tgfb1i1), and their interactions in injured liver will be confirmed by experiments.

In conclusion, based on the protein spectrum data from HPLC-MS, 80 upregulated and 67 downregulated DEPs were identified in the established rat liver injury model. The immunity-related Stat3 and some proteins (e.g. Junb, Fgg, Hmox1, Serpina3c and Tgfb1i1) interacting with it may be likely associated with liver injury. These results will be further verified and are expected to provide novel information for the diagnosis and therapy of liver injury.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (No. 814-41105 and No. 81360673), Inner Mongolia Autonomous Region Science and Technology Major Project (No. GCY20150833) and Doctoral Startup Fund of Inner Mongolia National University (No. BS413).

Disclosure of conflict of interest

None.

Address correspondence to: Bagenna Bao, School of Mongolian Medicine and Pharmaceutical Sciences, Inner Mongolia University for The Nationalities, 996, Xi La Mu Lun Road, Horqin District, Tonglia 028000, Inner Mongolia, P. R. China. Tel: +86-475-8314242; Fax: +86-475-8314242; E-mail: baobagenna68@hotmail.com; Tuul Khalzaabaast, College of Mongolian Medicine, Inner Mongolia University for The Nationalities, 996, Xi La Mu Lun Road, Horqin District, Tonglia 028000, Inner Mongolia, P. R. China. Tel: +86-475-8314242; Fax: +86-475-8314242; E-mail: 2217533243@qq.com

References


Protein spectrum in liver injured rat


