The aim of the present study was to examine differentially expressed proteome profiles for candidate biomarkers in peripheral blood mononuclear cells (PBMCs) of liver failure (LF) patients. Ten patients were diagnosed as LF and 10 age- and gender-matched subjects were recruited as healthy controls. Isobaric tags for relative and absolute quantitation (iTRAQ)-based quantitative proteomic technology is efficiently applicable for identification and relative quantitation of the proteomes of PBMCs. Eight-plex iTRAQ coupled with strong cation exchange chromatography, and liquid chromatography coupled with tandem mass spectrometry were used to analyze total proteins in LF patients and healthy control subjects. Molecular variations were detected using the iTRAQ method, and western blotting was used to verify the results. LF is a complex type of medical emergency that evolves following a catastrophic insult to the liver, and its outcome remains the most ominous of all gastroenterologic diseases. Serious complications tend to occur during the course of the disease and further exacerbate the problems. Using the iTRAQ method, differentially expressed proteome profiles of LF patients were determined. In the present study, 627 proteins with different expression levels were identified in LF patients compared with the control subjects; with 409 proteins upregulated and 218 proteins downregulated. Among them, four proteins were significantly differentially expressed; acylaminoacyl-peptide hydrolase and WW domain binding protein 2 were upregulated, and resistin and tubulin β 2A class Ia were downregulated. These proteins demonstrated differences in their expression levels compared with other proteins with normal expression levels and the significant positive correlation with LF. The western blot results were consistent with the results from iTRAQ. Thus, investigation of the molecular mechanism of the proteins involved in LF may facilitate an improved understanding of the pathogenesis of LF and elucidation of novel biomarker candidates.

Introduction

Liver failure (LF) is a complex medical emergency that evolves following a catastrophic insult to the liver with an outcome that remains the most ominous of all the gastroenterologic diseases. LF is severe liver damage resulting from various factors, which cause obstruction or decompensation of function, such as composition, detoxification, drainage and biotransformation. Various clinical syndromes appear, including the obstruction of coagulation mechanisms, icterus, hepatic encephalopathy and ascites. Serious complications, such as hepatic encephalopathy and renal inadequacy tend to occur during the course of the disease and further exacerbate clinical syndromes. It is conventionally defined by an arterial oxygen tension (P\textsubscript{a}O\textsubscript{2}) <8.0 kPa (60 mmHg) and/or an arterial carbon dioxide tension (P\textsubscript{a}CO\textsubscript{2}) >6.0 kPa (45 mmHg), and a serum bilirubin level ≥12.0 mg/dl. The prognosis of patients with severe liver injury, particularly with LF, largely depends upon the regenerative capacity of hepatocytes during comprehensive treatment. Liver transplantation improves survival and quality of life. However, treatment is futile in certain patients, but it is difficult to identify these patients a priori. In clinical practice, serum α-fetoprotein is often used as a predictive biomarker for monitoring the prognosis of patients with LF, as it reflects the regeneration of hepatocytes in response to liver injury (1-3). Although the most important function of telomerase is associated with cell proliferation and regeneration, to the best of our knowledge, there are no studies regarding the association of the prognosis of LF and telomerase.
activation. Thus, identification of molecular markers is required and novel treatments against this disease must be developed. In addition, improved non-invasive methods of detecting LF are urgently required in order to influence the survival of the increasing numbers of individuals affected by this disease.

The precise molecular pathogenic mechanism of LF remains unknown. Development of biomarkers for a deeper understanding of LF pathogenesis and improving diagnosis, prognosis, and treatment remains one of the main goals and challenges in LF research. Biomarkers within the blood and urine reflect the status and possible future progression of disease (4). Aberrant functions of the lymphocytic regulatory pathway are extensively involved in the pathological mechanism of certain diseases (5); therefore, peripheral blood mononuclear cells (PBMCs) are an attractive sample source in such studies. Proteomic analysis is a research method that catalogs all of the proteins within cells and organisms. Recent advancements in quantitative and large-scale proteomic methods may be used to optimize the clinical application of biomarkers (6). Furthermore, advancements of proteomic techniques contribute to the identification of clinically useful biomarkers and clarify the molecular mechanisms of disease pathogenesis using body fluids, such as serum, as well as tissue samples and cultured cells.

Proteomics analysis is a powerful technology used in a myriad of studies, including those focused on liver diseases (7-11). The isobaric tags for relative and absolute quantitation (iTRAQ) method allows a more comprehensive analysis. This method has a high sensitivity and it is possible to detect low-abundance proteins. iTRAQ has increasingly been applied in biomarker research in various sample sources for various disease states (12-14). Charlton et al (15) compared the protein expression profiles in four groups of liver tissue samples from obese patients using the combination of iTRAQ with liquid chromatography (LC)-mass spectrometry (MS) analysis. The isobaric tags for relative and absolute quantitation (iTRAQ) method allows a more comprehensive analysis. This method has a high sensitivity and it is possible to detect low-abundance proteins. iTRAQ has increasingly been applied in biomarker research in various sample sources for various disease states (12-14). The authors identified a total of 1,362 hepatic-expressed proteins, and identified two important proteins. Niu et al performed various in vitro proteomic investigations of Hepatitis B virus (HBV)-infected HepG2 hepatoma cells to evaluate the protein changes associated with the virus infection. Using the combined methods of iTRAQ with 2D-LC-MS, the authors compared the protein expression in non-infected HepG2 with that in HBV-infected HepG2 cells to identify various proteins that were downregulated in the HBV-infected cells, including S100 calcium-binding protein A6 and Annexin A2 (16,17).

In the present study, iTRAQ technology was used to analyze the total proteins in PBMCs of LF patients. The aim was to identify the differences in PBMC protein levels that were closely associated with the progression of LF. Further investigation into the molecular mechanism of the proteins involved may improve understanding of the pathogenesis of LF and facilitate development of novel approaches to diagnose and treat LF.

**Materials and methods**

*Main reagents.* Triton X-100 was purchased from GE Healthcare (Waukesha, WI, USA). Triethylammonium bicarbonate buffer was acquired from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany). ZipTip Pipette Tips and Milli-Q water were obtained from EMD Millipore (Billerica, MA, USA). The iTRAQ Reagent-8 Plex Multiplex kit was acquired from Applied Biosystems (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and Strata-X 33 Polymeric Reversed Phase was purchased from Phenomenex (Los Angeles, CA, USA). All other reagents were acquired from commercial sources.

*Patients and healthy controls.* Ten patients (6 male and 4 female; aged 23-57 years) were diagnosed as LF between January and December 2014, and 10 age- and gender-matched subjects were recruited as healthy controls. HBV-associated LF refers to patients with LF caused by chronic HBV infection. The 10 patients and 10 healthy control subjects were from Shenzhen People's Hospital (Shenzhen, China). The diagnosis of LF was confirmed by pathologic diagnosis and clinical evidence.

The control subjects were recruited and a general health checkup program confirmed that there was no clinical evidence of LF. All participants were informed of their participation rights and written informed consent was obtained. The present study was performed in accordance with the Helsinki Declaration and approved by the Regional Ethics Committee.

*PBMC isolation, protein extraction and quantitation.* One 10-ml fasting venous blood sample was collected in heparinized vacutainers from each enrolled subject. PBMCs were isolated with lymphocyte-H medium (Cedarlane Labs, Hornby, ON, Canada) according to the manufacturer's instruction. The total protein of PBMCs was extracted, and their concentration was measured using a BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's instruction. The proteins in the supernatant were maintained at -80°C for further analysis.

*iTRAQ labeling and strong cation exchange (SCX) chromatography fractionation.* Total protein (100 µg) from the PBMCs of the 10 LF patients and 10 healthy control subjects was digested separately with Trypsin Gold (Promega Corporation, Madison, WI, USA) with the ratio of protein:trypsin 30:1 at 37°C for 16 h. Following trypsin digestion, peptides were dried by vacuum centrifugation at 2,000 x g at room temperature for 10 min. Peptides were reconstituted in 0.5 M triethylammonium bicarbonate buffer and processed according to the manufacturer's protocol for the 8-plex iTRAQ reagent. Briefly, one unit of iTRAQ reagent was thawed and reconstituted in 24 µl isopropanol. Samples were labeled with the iTRAQ tags as follows: Sample 113 and sample 115. The peptides were labeled with the isobaric tags and incubated at room temperature for 2 h. The labeled peptide mixtures were then pooled and dried by vacuum centrifugation at 2,000 x g at room temperature for 10 min.

SCX chromatography was performed with a LC-20AB high-performance LC (HPLC) Pump system (Shimadzu Corp., Kyoto, Japan). The iTRAQ-labeled peptide mixtures were reconstituted with 4 ml buffer A (25 mM NaH₂PO₄ in 25% ACN, pH 2.7) and loaded onto a 4.6x250 mm Ultemrex SCX column containing 5-µm particles (Phenomenex). The peptides were eluted at a flow rate of 1 ml/min with a gradient of buffer A for 10 min, 5-60% buffer B (25 mM NaH₂PO₄, 1 M
LC-MS/MS analysis based on Q-EXACTIVE. Each fraction was resuspended in buffer A (2% ACN, 0.1% FA) and centrifuged at 20,000 x g for 10 min at 4°C, then the final concentration of peptide was ~0.5 μg/μl on average. Supernatant (10 μl) was then loaded onto an LC-20AD nano HPLC (Shimadzu Corp., Kyoto, Japan) by the autosampler onto a 2-cm C18 trap column (inner diameter, 200 μm). Then, the peptides were eluted onto a 10-cm analytical C18 column (inner diameter, 75 μm) packed in-house. The samples were loaded at 8 μl/min for 4 min, then the 44-min gradient was run at 300 nl/min starting from 2 to 35% solvent B (98% ACN, 0.1% fatty acid), followed by a 2-min linear gradient to 80% and maintenance at 80% solvent B for 4 min, and finally returning to 5% in 1 min.

The peptides were subjected to nanoelectrospray ionization followed by MS/MS in a Q-EXACTIVE (Thermo Fisher Scientific, Inc.) coupled online to the HPLC. Intact peptides were detected in the orbitrap (Thermo Fisher Scientific, Inc.) at a resolution of 70,000. Peptides were selected for MS/MS using high-energy collision dissociation operating mode with a normalized collision energy setting of 27.0; ion fragments were detected in the orbitrap at a resolution of 17,500. A data-dependent procedure that alternated between one MS scan followed by 15 MS/MS scans was applied for the 15 most abundant precursor ions above a threshold ion count of 20,000 scans in the orbitrap (Thermo Fisher Scientific, Inc.) coupled online to the HPLC. Intact peptides were selected for MS/MS using high-energy collision dissociation operating mode with a normalized collision energy setting of 27.0; ion fragments were detected in the orbitrap at a resolution of 17,500.

Results

Protein expression profile. Compared with the control group, a total of 627 differently expressed proteins were detected, of which 409 proteins showed increased expression levels and 218 proteins showed decreased expression levels in LF (Table I). The protein ratio distribution of these proteins is illustrated in Fig. 1. Relative quantification of proteins was based on the ratio of peak areas from the MS/MS spectra, and the m/z of LF patients and control subjects were involved in the present study.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. The functions of the differently expressed proteins were analyzed using the GO and KEGG pathways annotation system. The proteins produced a total of 420 GO terms in the LF group (Table II), including 284 in biological process, 74 in cellular component and 62 in molecular function. The results indicate that a set of highly abundant and significantly differentially expressed proteins may promote the progression of LF patients. In addition, 14 KEGG pathways of the differently expressed proteins in LF were obtained (Table III).

Discussion

Protein quantification has become an important and, in many cases, critical component of modern MS-based proteomic research (19,20). Proteomics is the term used for exhaustive analysis of protein structure and function. It is useful for elucidation of the pathology and identification of disease markers for liver diseases. PBMCs are often used as clinical samples, rather than tissue, as less invasive methods may be used to
### Table I. LF proteome.

#### A. Top 30 increased proteins in LF

| No. | Accession | Description | Ratio of LF to control |
|-----|-----------|-------------|------------------------|
| 1   | sp|P22392|NDKB_HUMAN | Nucleoside diphosphate kinase B | 3.953 |
| 2   | sp|Q9UM07|PADI4_HUMAN | Protein-arginine deiminase type-4 | 3.509 |
| 3   | sp|Q9QC6B|BZW1_MOUSE | Basic leucine zipper and W2 domain-containing protein 1 | 3.431 |
| 4   | sp|Q96DA6|TIM14_HUMAN | Mitochondrial import inner membrane translocase subunit TIM14 | 3.297 |
| 5   | sp|P57901|HXK3_HUMAN | Hexokinase-3 | 2.957 |
| 6   | sp|P80188|NGAL_HUMAN | Neutrophil gelatinase-associated lipocalin | 2.928 |
| 7   | sp|P25774|CATS_HUMAN | Cathepsin S | 2.8 |
| 8   | sp|P12429|ANXA3_HUMAN | Annexin A3 | 2.631 |
| 9   | sp|P37837|TALDO_HUMAN | Transaldolase | 2.595 |
| 10  | sp|P41218|MNDA_HUMAN | Myeloid cell nuclear differentiation antigen | 2.55 |
| 11  | sp|Q9ULZ3|ASC_HUMAN | Apoptosis-associated speck-like protein containing a CARD | 2.514 |
| 12  | sp|Q4R6V2|TCPE_MACFA | T-complex protein 1 subunit epsilon | 2.362 |
| 13  | sp|P26583|HMGB2_HUMAN | High mobility group protein B2 | 2.361 |
| 14  | sp|Q9UBW5|TIM14_HUMAN | Bridging integrator 2 | 2.355 |
| 15  | sp|Q6P4A8|PLBL1_HUMAN | Phospholipase B-like 1 | 2.339 |
| 16  | sp|A6NI72|NCF1B_HUMAN | Putative neutrophil cytosol factor 1B | 2.338 |
| 17  | sp|P39687|AN32A_HUMAN | Acidic leucine-rich nuclear phosphoprotein 32 family member A | 2.295 |
| 18  | sp|Q92688|AN32B_HUMAN | Acidic leucine-rich nuclear phosphoprotein 32 family member B | 2.295 |
| 19  | sp|P61586|RHOA_HUMAN | Transforming protein RhoA | 2.292 |
| 20  | sp|P20700|LMNB1_HUMAN | Lamin-B1 | 2.249 |
| 21  | sp|O75962|TRIO_HUMAN | Triple functional domain protein | 2.233 |
| 22  | sp|Q99055|CSN5_HUMAN | COP9 signalosome complex subunit 5 | 2.201 |
| 23  | sp|P18433|PTPRA_HUMAN | Receptor-type tyrosine-protein phosphatase α | 2.187 |
| 24  | sp|Q8BL97|SRSF7_MOUSE | Serine/arginine-rich splicing factor 7 | 2.186 |
| 25  | sp|P85951|SPB10_HUMAN | Serpin B10 | 2.171 |
| 26  | sp|P18402|EMD_HUMAN | Emerin | 2.17 |
| 27  | sp|O96006|ZBED1_HUMAN | Zinc finger BED domain-containing protein 1 | 2.162 |
| 28  | sp|P96686|CATH_HUMAN | Pro-cathepsin H | 2.146 |
| 29  | sp|P50395|GDIH_HUMAN | Rab GDP dissociation inhibitor β | 2.1 |
| 30  | sp|O73777|IF4G2_CHICK | Eukaryotic translation initiation factor 4 γ 2 (Fragment) | 2.091 |

#### B. Top 30 decreased proteins in LF

| No. | Accession | Description | Ratio of LF to control |
|-----|-----------|-------------|------------------------|
| 1   | sp|Q9Y2R4|DDX52_HUMAN | Probable ATP-dependent RNA helicase DDX52 | 0.078 |
| 2   | sp|Q75015|FCG3B_HUMAN | Low affinity immunoglobulin gamma Fc region receptor III-B | 0.211 |
| 3   | sp|P12236|ADT3_HUMAN | ADP/ATP translocase 3 | 0.277 |
| 4   | sp|Q9NTG7|SIR3_HUMAN | NAD-dependent protein deacetylase sirtuin-3, mitochondrial | 0.298 |
| 5   | sp|P14222|PERF_HUMAN | Perforin-1 | 0.346 |
| 6   | sp|P20718|GRAH_HUMAN | Granzyme H | 0.39 |
| 7   | sp|P04264|K2C1_HUMAN | Keratin, type II cytoskeletal 1 | 0.419 |
| 8   | sp|P19086|GNAR_HUMAN | Guanine nucleotide-binding protein G(z) subunit α | 0.433 |
| 9   | sp|P11666|GTR1_HUMAN | Solute carrier family 2, facilitated glucose transporter member 1 | 0.434 |
| 10  | sp|Q969X1|LFG3_HUMAN | Protein lifeguard 3 | 0.437 |
| 11  | sp|Q15050|RRS1_HUMAN | Ribosome biogenesis regulatory protein homolog | 0.442 |
| 12  | sp|P12544|GRAA_HUMAN | Granzyme A | 0.448 |
| 13  | sp|P14209|CD99_HUMAN | CD99 antigen | 0.45 |
obtain them. If a biomarker associated with the pathology, disease progression or efficacy of treatment is identified in PBMCs, it may be easily applied for early or differential diagnosis of diseases. Of these, iTRAQ, which enables the parallel comparison of protein abundance by measuring the peak intensities of reporter ions released from iTRAQ-tagged peptides, has the potential to be a key tool in the area of quantitative proteomic study. In the current study, iTRAQ technology was adopted to quantitatively analyze the proteomics of PBMCs from LF patients and healthy control subjects. As a result, 627 proteins involving different biological functions and cellular locations were identified. Among these proteins, four proteins were significantly differentially expressed; APEH and WBP2 were upregulated, and resistin and TUBB2A were downregulated. It provided additional proof that the iTRAQ technique accurately quantifies relative changes in protein abundance of PBMCs, which has been demonstrated to be useful in detecting pathological stages or prognosis in certain diseases, such as osteoarthritis (21,22).

Resistin is cysteine-rich protein belonging to the RELM family. The genetic structure of the Retn gene varies between mammals and the similarity in the coding sequence ranges from ~60% for rodents to 80% for livestock (23). The expression of this gene in rodents occurs predominantly in mature adipocytes, although it was also identified in other tissues. Retn is considered to be a factor linking obesity and insulin resistance. In obesity, its expression increases, leading to enhanced resistance of tissues to insulin (24).

TUBB2A is thought to comprise ~30% of all β-tubulin within the brain (25) and contributes to the growing list of tubulin gene mutations that are associated with impaired brain development in humans. Increased expression levels of TUBB2A have been correlated with decreased drug sensitivity in paclitaxel-resistant cell lines (26). A previous study demonstrated the potential of a model for gene perturbation studies by demonstrating that decreased expression levels of TUBB2A result in significantly increased sensitivity of neurons to paclitaxel (27).

Table I. Continued

| No. | Accession | Description | Ratio of LF to control |
|-----|-----------|-------------|-----------------------|
| 14  | sp|P07996|TSP1_HUMAN | Thrombospondin-1 | 0.454 |
| 15  | sp|P05106|ITB3_HUMAN | Integrin β-3 | 0.454 |
| 16  | sp|P02788|TRFL_HUMAN | Lactotransferrin | 0.479 |
| 17  | sp|P35527|K1C9_HUMAN | Keratin, type I cytoskeletal 9 | 0.487 |
| 18  | sp|O15533|TPSN_HUMAN | Tapasin | 0.491 |
| 19  | sp|O60704|ITPST2_HUMAN | Protein-tyrosine sulfotransferase 2 | 0.504 |
| 20  | sp|Q9BVC6|ITM109_HUMAN | Transmembrane protein 109 | 0.506 |
| 21  | sp|P57840|SYUA_HUMAN | α-synuclein | 0.511 |
| 22  | sp|Q08AF3|SLFN5_HUMAN | Schlafen family member 5 | 0.514 |
| 23  | sp|P16109|ILYAM3_HUMAN | P-selectin | 0.517 |
| 24  | sp|P18871|IGHCM_HUMAN | Ig μ chain C region | 0.523 |
| 25  | sp|P50336|IPOX_HUMAN | Protoporphyrinogen oxidase | 0.529 |
| 26  | sp|P02788|TRFL_HUMAN | Lactotransferrin | 0.543 |
| 27  | sp|P68872|HBB_PANPA | Hemoglobin subunit β | 0.548 |
| 28  | sp|Q9Y6W5|IWASF2_HUMAN | Wiskott-Aldrich syndrome protein family member 2 | 0.549 |
| 29  | sp|P18428|LB_P_HUMAN | Lipopolysaccharide-binding protein | 0.556 |
| 30  | sp|Q99798|ACON_HUMAN | Aconitate hydratase, mitochondrial | 0.556 |

Ratios with P<0.05 and fold-change >1.2 were considered to be statistically significant. The top 30 proteins that were increased or decreased in LF according to iTRAQ were extracted. LF, liver failure; iTRAQ, isobaric tags for relative and absolute quantitation.

Figure 1. Protein ratio distribution of differentially expressed proteins in liver failure. Green, downregulated differently expressed proteins; red, upregulated differently expressed proteins.
The role of WBP-2 is as a coactivator for estrogen receptor and progesterone receptor transactivation pathways (28). APEH has been postulated to serve as a key regulator of N-terminal acetylated proteins (29). As >80% of proteins in human cells are N-terminal acetylated (30,31) and protein acetylation is implicated in a variety of essential cellular pathways (32), it is feasible that APEH is involved in these processes. APEH, one of the four members of the prolyl oligopeptidase class, catalyses the removal of N-acetylated amino acids from acetylated peptides and it has been postulated to be key in protein degradation machinery. Disruption of protein turnover has been established as an effective strategy to downregulate the ubiquitin proteasome system and as a promising approach in anticancer therapy. APEH may be an upstream modulator of the proteasome (33).

To establish the biological roles of the proteins from LF, GO enrichment and KEGG pathway analyses were performed. GO
categories were separated into three groups: Molecular function, biological process and cellular component. The present study identified GO terms for molecular function significantly enriched in protein binding (GO:0005515; P=0.02145) involving TUBB2A and WBP2, and for cellular component, the enriched GO terms were cytoplasm (GO:0005737; P=0.02634) involving APEH and TUBB2A. KEGG analysis was performed with P<0.05 as the criteria for significant pathway identification. The significant pathway in the KEGG analysis was identified as phagosome (P=0.02839) involving TUBB2A.

Phagocytosis is the process of a cell taking in relatively large particles, and is a central mechanism in tissue remodeling, inflammation and defense against infectious agents. A phagosome is formed when the specific receptors on the phagocyte surface recognize ligands on the particle surface. Following formation, nascent phagosomes progressively acquire digestive characteristics.

In conclusion, proteomic technologies based on MS have been developed, and the reliability of these technologies continues to improve. Such advancements in proteomic techniques may contribute to the identification of clinically useful biomarkers and the elucidation of the molecular mechanisms involved in disease pathogenesis. Therefore, a more sensitive detection system to search for biomarkers is required, and this may allow clinically useful markers for all liver diseases to be identified. Proteins are assumed to be key molecules that define the characteristics and dynamics of cells, and control biological reactions. Therefore, investigation of changes in protein expression levels is particularly important in understanding disease pathology. A limitation of the current study is that it did not discuss each of the candidate proteins in detail. The aim of this preliminary study was to focus on delineating primary comparative protein profiles of LF patients and healthy control subjects using iTRAQ technology. In future, a large-scale clinical study is required to investigate useful biomarkers of LF. This may result in a novel method for diagnosing LF. In addition, the current study demonstrates the potential application of iTRAQ-based quantitative proteomics for identifying protein changes and detecting notable biomarker candidates in certain diseases. Thus, identification and evaluation of an easily measurable biomarker is imperative. A combination of conventional markers with newly identified markers, the variation of which was confirmed in the present study, may improve diagnosis of the LF disease state and the capacity for prognosis.

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