Differential proteomics analysis of liver failure in peripheral blood mononuclear cells using isobaric tags for relative and absolute quantitation

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Abstract. 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 IIa 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.

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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, detoxifıcation, 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_aO_2) <8.0 kPa (60 mmHg) and/or an arterial carbon dioxide tension (P_aCO_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 a 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)/MS. 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/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 triethyl-ammonium 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 NaH_2PO_4 in 25% ACN, pH 2.7) and loaded onto a 4.6x250 mm Ultremex 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 NaH_2PO_4, 1 M

KCl in 25% ACN, pH 2.7) for 27 min and 60-100% buffer B for 1 min. The system was then maintained at 100% buffer B for 1 min before equilibrating with buffer A for 10 min prior to the next injection. Elution was monitored by measuring the absorbance at 214 nm with ultraviolet-visible spectrophotometry, and fractions were collected at 1-min intervals. The eluted peptides were pooled into 20 fractions, desalted with a Strata X C18 column (Phenomenex) and vacuum-dried.

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, the final concentration of peptide was ~0.5 μg/μl on average. Supernatant (10 μl) was 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 in the MS survey scan with a subsequent dynamic exclusion duration of 15 sec. The electrospray voltage applied was 1.6 kV. Automatic gain control (AGC) was used to optimize the spectra generated by the orbitrap. The AGC target for full MS was 3e6 and 1e5 for MS2. For the MS scans, the mass to charge ratio (m/z) scan range was 350-2,000 Da, and for the MS2 scans, the m/z scan range was 100-1,800.

Western blot analysis. The protein abundance of the pooled samples previously analyzed by iTRAQ LC-MS/MS was confirmed essentially as previously described using western blotting (18). Four protein samples (35 μg) were added into electrophoretic buffer containing β-mercaptoethanol prior to SDS-PAGE. GAPDH (Kangcheng, Shanghai, China) served as a loading control. The primary antibody (dilution, 1:250; cat. no. ab89835) for ADP-ribosylation factor 1 was obtained from Abcam (Cambidge, MA, USA) and the peroxidase-conjugated goat anti-rabbit IgG secondary antibody (dilution, 1:10,000) was obtained from SouthernBiotech (Birmingham, AL, USA; cat. no. A21020). The band intensity of western blot analysis was repeated three times using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Protein identification was performed using Mascot version 2.3.02 (Matrix Science, London, UK). Peptide sequences were searched against the non-redundant National Center for Biotechnology Information database. For protein quantitation, it was required that a protein contained at least two unique peptides. The quantitative protein ratios were weighted and normalized by the median ratio in Mascot. Only ratios with $P<0.05$ and fold-change >1.2 were considered to be statistically significant. Search criteria were set to permit a maximum of 1 missed cleavage. The following peptide modifications were also allowed: Gln→pyro-Glu, iTRAQ 8plex, Phospho. Automatic isotope correction was performed using the two software packages using the values supplied with the Applied Biosystems reagents. The PANTHER database (http://www.pantherdb.org/panther/) was then used to establish the molecular function, biological process and signaling pathway associated with each individual protein. GO and KEGG pathway mapping were performed by web-accessible DAVID annotation system (version 6.7; https://david-d.ncifcrf.gov/).

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).

Western blot analysis. Four proteins, acylaminoacyl-peptide hydrolase (APEH; 1.309), WW domain binding protein 2 (WBP2; 1.385), resistin (0.747) and tubulin β 2A class IIa (TUBB2A; 0.764), were selected from the 627 proteins with variable expression levels. These differently expressed proteins between the LF and control groups were verified by western blotting (Table IV and Fig. 2), which indicated a similar relative expression level when compared with the iTRAQ LC-MS/MS analysis.

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|Q9CQC6|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|P52790|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|BIN2_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|Q92905|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|P48595|SPB10_HUMAN	Serpin B10	2.171
26	sp|P50402|EMD_HUMAN	Emerin	2.17
27	sp|O96006|ZBED1_HUMAN	Zinc finger BED domain-containing protein 1	2.162
28	sp|P09668|CATH_HUMAN	Pro-cathepsin H	2.146
29	sp|P50395|GDIB_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|O75015|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|GNAZ_HUMAN	Guanine nucleotide-binding protein G(z) subunit α	0.433
9	sp|P11166|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

Table I. Continued

B, Top 30 decreased proteins in LF

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|TPST2_HUMAN	Protein-tyrosine sulfotransferase 2	0.504
20	sp|Q9BVC6|TM109_HUMAN	Transmembrane protein 109	0.506
21	sp|P37840|SYUA_HUMAN	α-synuclein	0.511
22	sp|Q08AF3|SLFN5_HUMAN	Schlafen family member 5	0.514
23	sp|P16109|LYAM3_HUMAN	P-selectin	0.517
24	sp|P01871|IGHM_HUMAN	Ig μ chain C region	0.523
25	sp|P50336|PPOX_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|WASF2_HUMAN	Wiskott-Aldrich syndrome protein family member 2	0.549
29	sp|P18428|LBP_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.

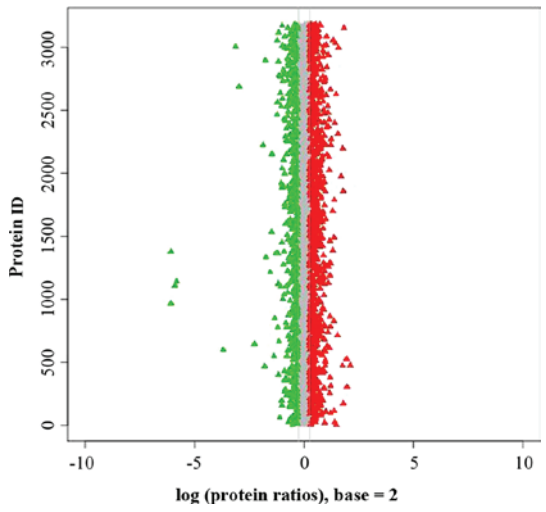

Figure 1. Protein ratio distribution of differentially expressed proteins in liver failure. Green, downregulated differently expressed proteins; red, upregulated differently expressed proteins.

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 II. Up- and downregulated protein annotation terms of the GO molecular function, cellular component and biological process categories in LF.

Term	P-value	Term	P-value
Biological process			
Transport	6.41E-05	Multi-organism process	0.001086326
Establishment of localization	6.58E-05	DNA damage response, signal transduction by p53 class mediator	0.001163164
Immune system process	0.000134159	Vesicle-mediated transport	0.001412972
Immune response	0.000164528	Intracellular transport	0.001540664
Establishment of localization in cell	0.000176366	Release of sequestered calcium ion into cytosol	0.001593621
Negative regulation of molecular function	0.000205286	Regulation of sequestering of calcium ion	0.001593621
Negative regulation of catalytic activity	0.000304827	Negative regulation of sequestering of calcium ion	0.001593621
Localization	0.000494539	Cytosolic calcium ion transport	0.001593621
Vesicle fusion	0.00085815	Calcium ion transport into cytosol	0.001593621
Cellular localization	0.000951991	Signal transduction by p53 class mediator	0.001850301
Molecular function			
Cation transmembrane transporter activity	0.000354884	endopeptidase regulator activity	0.006225752
Hydrogen ion transmembrane transporter activity	0.001472303	Immunoglobulin binding	0.006472871
Inorganic cation transmembrane transporter activity	0.002536343	Substrate-specific transmembrane transporter activity	0.006837942
Peptidase regulator activity	0.002777008	Endopeptidase inhibitor activity	0.007710339
Transporter activity	0.003623169	Peptidase inhibitor activity	0.007710339
Peptide transporter activity	0.004093554	Monosaccharide binding	0.008311587
Amide transmembrane transporter activity	0.004093554	Ubiquinol-cytochrome-c reductase activity	0.008525874
Enzyme inhibitor activity	0.004329426	Ferric iron binding	0.008525874
Substrate-specific transporter activity	0.004897002	Oxidoreductase activity, acting on diphenols and related substances as donors	0.008525874
Ion transmembrane transporter activity	0.005584917	Oxidoreductase activity, acting on diphenols and related substances as donors, cytochrome as acceptor	0.008525874
Cellular component			
Cytoplasmic vesicle	0.000318	Phagocytic vesicle	0.003696
Vesicle	0.000409	endosomal part	0.005078
Cytoplasmic membrane-bounded vesicle	0.000483	Proteasome complex	0.005818
Secretory granule	0.000497	Endosome	0.006978
Cytoplasmic vesicle part	0.000632	Proteasome core complex, alpha-subunit complex	0.008175
Membrane-bounded vesicle	0.000705	TAP complex	0.00878
Platelet alpha granule	0.00168	Cytoplasmic vesicle membrane	0.009488
Secondary lysosome	0.002441	Endosome membrane	0.010481
Phagolysosome	0.002441	Vesicle membrane	0.011522
Endocytic vesicle	0.002743	Organelle envelope lumen	0.011583

P<0.05 was considered to be statistically significant. The top 20 GO terms were listed in LF according to molecular function, cellular component and biological process. GO, Gene Ontology; LF, liver failure.

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

Table III. The Kyoto Encyclopedia of Genes and Genomes pathways of the differently expressed proteins in liver failure.

Pathway	P-value	Pathway ID
Spliceosome	0.0004265046	ko03040
Cardiac muscle contraction	0.0006583187	ko04260
Proteasome	0.002674917	ko03050
Oxidative phosphorylation	0.004688983	ko00190
Parkinson's disease	0.0061224	ko05012
Valine, leucine and isoleucine biosynthesis	0.008369825	ko00290
Malaria	0.01015188	ko05144
Pathogenic *Escherichia coli* infection	0.0161563	ko05130
Leukocyte transendothelial migration	0.01934782	ko04670
Alzheimer's disease	0.01948298	ko05010
Glycolysis/gluconeogenesis	0.02570021	ko00010
Osteoclast differentiation	0.02836594	ko04380
Phagosome	0.02839378	ko04145
Galactose metabolism	0.0475115	ko00052

P<0.05 was considered to indicate a statistically significant difference.

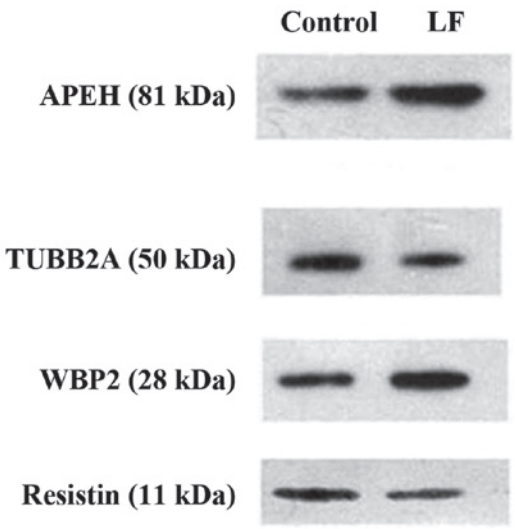

Figure 2. Western blot analysis of APEH, WBP2, Resistin and TUBB2A in the LF and control groups. APEH, acylaminoacyl-peptide hydrolase; WBP2, WW domain binding protein 2; TUBB2A, tubulin β 2A class IIa; LF, liver failure.

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.

Table IV. Western blot analysis of APEH, WBP2, resistin and TUBB2A in the LF and control groups.

Name	Control (relative expression)	LF (relative expression)
Resistin	1.000	0.715
APEH	1.000	1.297
TUBB2A	1.000	0.738
WBP2	1.000	1.072

APEH, acylaminoacyl-peptide hydrolase; WBP2, WW domain binding protein 2; TUBB2A, tubulin β 2A class IIa; LF, liver failure.

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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