iTRAQ-based proteomic analysis of hepatic tissues from patients with hepatitis B virus-induced acute-on-chronic liver failure

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Abstract. The pathogenesis of hepatitis B virus (HBV)-induced acute-on-chronic liver failure (ACLF), a serious and prevalent medical condition, is not clear, particularly with regard to which proteins are expressed in the course of the disease. The aim of the present study was to identify the differences in hepatic tissue protein expression between normal human subjects and patients with ACLF using isobaric tags for relative and absolute quantification (iTRAQ)-based proteomic analysis and to verify the results using western blot analysis. The iTRAQ method was used to analyze the protein contents of hepatic tissue samples from 3 patients with HBV-induced ACLF and 3 normal healthy subjects. The results were verified by subjecting the hepatic tissues from 2 patients with HBV-induced ACLF and 4 healthy subjects to western blot analysis. In total, 57 proteins with ≥1.5-fold differences between patients with HBV-induced ACLF and healthy subjects were identified using iTRAQ. Among these 57 proteins, 4 with the most marked differences in their expression and the most significant association with liver disease were selected to be verified through western blot analysis: Keratin, type-I cytoskeletal 19; α-1-acid glycoprotein 1 (α1-AGP); carbonic anhydrase-1; and serpin peptidase inhibitor and clade A (α-1 anti proteinase, antitrypsin) member 1 (SERPINA1). The results of the western blot analyses were nearly identical to the iTRAQ results. Identifying the differences in liver protein expression in patients with HBV-induced ACLF may provide a basis for studies on the pathogenesis of ACLF. Future studies should focus particularly on α1-AGP, carbonic anhydrase 1 and SERPINA1.

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Abbreviations: iTRAQ, isobaric tag for relative and absolute quantification; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; ACLF, acute-on-chronic liver failure; CHB, chronic hepatitis B; HBsAg, hepatitis B surface antigen; HBsAb, hepatitis B surface antibody; HBeAg, hepatitis B e antigen; HBeAb, hepatitis B e antibody; HBcAb, hepatitis B core antibody; HAV, hepatitis A virus; HCV, hepatitis C virus; HDV, hepatitis D virus; HEV, hepatitis E virus; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBIL, total billirubin; PT, prothrombin time; INR, international normalized ratio; BUN, blood urea nitrogen; CR, creatinine; MELD, model for end-stage liver disease; WBC, white blood cell; RBC, red blood cell; HB, hemoglobin; PLT, platelet

Key words: isobaric tag for relative and absolute quantification, acute-on-chronic liver failure, keratin, type I cytoskeletal 19, α-1-acid glycoprotein 1, carbonic anhydrase 1, serpin peptidase inhibitor and clade A (α-1 antiproteinase, antitrypsin), member 1

Introduction

Hepatitis B is a prevalent disease in China and the most common risk factor for liver cirrhosis and hepatocellular carcinoma (HCC). It has been reported that 7.18% of the Chinese population aged 1-59 years is seropositive for hepatitis B surface antigen (1). In China, there are ~93 million hepatitis B virus (HBV) carriers, 20 million of whom are patients with chronic hepatitis B (CHB) (2). Despite its high prevalence, the pathogenesis of acute-on-chronic liver failure (ACLF) remains unclear, particularly regarding the protein expression and regulatory processes that are involved.

Proteomic analysis is a powerful technological tool for investigations of human diseases, such as liver diseases (3-7). Isobaric tags for relative and absolute quantification (iTRAQ) is a quantitative method that has frequently been used in proteomic studies and is considered to exhibit a sensitivity that is equal to or greater than that of difference gel electrophoresis, a technique used to monitor the differences in proteomic profile between cells in different functional states (8). The iTRAQ method has been demonstrated to be effective and accurate in characterizing numerous diseases (4,5,9).

In the present study, the iTRAQ method was used to analyze the expression of various proteins in hepatic tissue extracted from patients with HBV-induced ACLF and from normal control subjects, and the iTRAQ results were verified

using western blot analysis. The aim of the study was to identify differences in protein expression that were closely associated with the pathogenesis of HBV-induced ACLF, in order to provide a basis for understanding the mechanisms underlying the pathogenesis of ACLF.

Materials and methods

Patients and specimens. All hepatic tissues were obtained from orthotopic liver transplantations performed in the Department of Hepatobiliary Surgery at the First Affiliated Hospital of Sun Yat-Sen University (Guangzhou, China). A total of 5 samples of normal hepatic tissue were extracted from whole-organ donor livers of adults that succumbed to circulatory failure. In addition, 7 samples of abnormal hepatic tissue were obtained from the resected livers of patients with HBV-induced ACLF. The diagnoses of HBV-induced ACLF were based on previously described criteria (10,11). Exclusion criteria included the following: Liver cirrhosis, diagnosed by B ultrasound and computed tomography; pregnancy; anti-viral or immunomodulatory therapy within 6 months; other factors causing active liver diseases, such as hepatitis A, C, D and E or autoimmune, drug-induced liver, alcoholic liver and inherited metabolic liver diseases; concomitant human immunodeficiency virus infection or congenital immune deficiency diseases; confirmed diagnosis of liver cancer or other malignancies; severe diabetes, autoimmune diseases or other major organ dysfunction; and concomitant infection or other serious complications.

Protein contents in 6 separate hepatic tissue samples were analyzed using iTRAQ analysis (Table I); 3 tissue samples were from normal hepatic tissue and 3 samples were from patients with HBV-induced ACLF. The differences in protein expression in a further 6 hepatic tissue samples were subsequently verified using western blot analysis (Table I); 2 of these samples were from normal hepatic tissues and 4 samples were from patients with HBV-induced ACLF.

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the appropriate institutional review committee of the Third Affiliated Hospital of Sun Yat Sen University (Guangzhou, China). Informed consent was obtained from all patients and healthy subjects prior to the initiation of the study.

Sample preparation and protein extraction. Frozen liver samples were stored at -80˚C prior to transfer to a liquid nitrogen pre-chilled mortar. Samples were ground into powder using liquid nitrogen. The powder was placed in a centrifuge tube and precipitated at -20˚C for 2 h by adding 10% trichloroacetic acid-ice acetone at a volume 10-times that of the volume of the powder. Samples were centrifuged at 20,000 x g at 4˚C for 30 min, and the pellet was collected following the removal of the supernatant. Cold acetone was then added to the pellet at 2-3-times the volume of the pellet, and the sample was precipitated at -20˚C for 30 min. The sample was subsequently centrifuged twice at 20,000 x g at 4˚C for 30 min, the pellet was air-dried and a lysis buffer, containing 1 mM phenylmethylsulfonyl fluoride, 2 mM EDTA and 10 mM dithiothreitol (DTT), was added. The sample was sonicated via ultrasound for 5 min in an ice-bath and centrifuged at 20,000 x g for 25 min. The resulting supernatant was the protein solution, which was quantified using the Bradford method (9).

Tissue digestion and iTRAQ reagent labeling. Equal 100-μg quantities of total protein from the liver tissue of patients with ACLF and control subjects were pooled separately for iTRAQ labeling, followed by sample alkylation. After adding 10 mM DTT, prepared by combining 10 μl DTT (1 M) and 990 μl NH_4HCO_3 (25 mM), the sample was incubated in a 56˚C water bath for 1 h, and then cooled to room temperature. Following the drying of the sample, 20 μl iodoacetamide (IAM; 55 mM), prepared by combining 55 μl IAM (1 M) and 945 μl NH_4HCO_3 (25 mM), was added immediately and the sample was placed in a dark chamber for 45 min. The sample was precipitated by adding 4-times the volume of acetone and incubating for 2 h, followed by centrifugation at 20,000 x g for 30 min. Following the removal of the supernatant to ensure minimum residual acetone, the sample was allowed to dry to 70% weight. The protein was dissolved by adding 50% tetraethylammonium bromide (TEAB) + 0.1% sodium dodecyl sulfate rapidly to the sample. The sample was replenished with 50% TEAB (9-times the volume of the sample), vortexed, mixed and then subjected to centrifugation at 1,000 x g for 30 sec at room temperature. Following alkylation, the sample was digested with trypsin protease (Trypsin Gold, Mass Spectrometry Grade; Promega Corp., Madison, WI, USA). In the alkylated sample, trypsin protease solution (1 μg/μl) was added at a ratio of 1:25 and mixed well. The digestion was incubated in a 37˚C water bath for 24 h, and the digested protease solution was then freeze-dried for subsequent iTRAQ labeling. The dried powder was resolved using 50 μl 50% TEAB and mixed well, prior to the addition of 70 μl isopropanol and further mixing. iTRAQ Labeling reagent (Applied Biosystems, Life Technologies, Foster City, CA, USA) was then added (group A reporter, 114; group B reporter, 116). Subsequent to vortexing for 10 sec, the sample was centrifuged at 1,000 x g for 30 sec at room temperature and incubated at room temperature for 2 h. The labeling peptides were mixed well for strong cation exchange (SCX) chromatography separation.

SCX chromatography. SCX chromatography separation was performed to remove the excess iTRAQ reagent and interfering substances for the mass analysis using an Agilent 1100 series high-performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, CA, USA). Labeled peptides were re-suspended in a Luna 5-μm SCX 100 Å HPLC column (250x4.60 mm; Phenomonex, Torrance, CA, USA) using the Agilent HPLC system. Buffer A consisted of 10 mM KH_2PO_4 and 25% acetonitrile (pH 3.0), and buffer B consisted of 10 mM KH_2PO_4, 2 M KCl and 25% acetonitrile (pH 3.0). The 60-min gradient comprised the following: 0.01-30 min, mobile phase with 100% buffer A elution to balance baseline and pressure; 30-31 min, mobile phase with 0-5% buffer B and 100-95% buffer A elution; 31-46 min, mobile phase with 5-30% buffer B and 95-70% buffer A elution; 46-51 min, mobile phase with 30-50% buffer B and 70-50% buffer A elution; 51-55 min, mobile phase with 50% buffer B and 50% buffer A elution; 55-60 min, mobile phase with 50-0% buffer B and 50-100% buffer A elution.

Table I. Clinical manifestations of 12 samples for iTRAQ (1-6) and western blot (7-12) analyses: 5 liver tissue samples from healthy humans and 7 from HBV-induced ACLF patients.

Parameter	1	2	3	4	5	6	7	8	9	10	11	12
Subject type	Normal	Normal	Normal	Patient	Patient	Patient	Normal	Normal	Patient	Patient	Patient	Patient
Gender	Male	Male	Female	Male	Male	Female	Male	Female	Male	Female	Male	Male
Age (years)	25	34	28	56	47	52	36	30	39	51	46	55
HBsAg (+/-)	-	-	-	+	+	+	-	-	+	+	+	+
HBsAb (+/-)	+	-	+	-	-	-	+	+	-	-	-	-
HBeAg (+/-)	-	-	-	-	+	-	-	-	-	+	+	+
HBeAb (+/-)	-	-	-	+	-	+	-	-	+	-	-	-
HBcAb (+/-)	-	-	-	+	+	+	-	-	+	+	+	+
Anti-HAV (+/-)	-	-	-	-	-	-	-	-	-	-	-	-
Anti-HCV (+/-)	-	-	-	-	-	-	-	-	-	-	-	-
Anti-HDV (+/-)	-	-	-	-	-	-	-	-	-	-	-	-
Anti-HEV (+/-)	-	-	-	-	-	-	-	-	-	-	-	-
AST (14.5-40 U/l)	33	24	22	87	55	67	21	15	26	78	101	58
ALT (3-35 U/l)	24	15	19	56	43	89	31	24	35	65	46	110
TBIL (4-23.9 μmol/l)	21.1	14.2	12.7	611.5	355.7	529.5	13.8	20.1	463.5	312.5	556.7	536.8
PT (11-14.5 sec)	11.3	11.7	12.0	32.5	27.8	29.2	12.3	11.1	25.5	35.2	36.1	32.3
INR	1.07	1.12	1.21	3.15	2.58	2.87	1.16	1.09	2.41	3.55	3.75	3.08
HBV DNA (IU/ml)	UD	UD	UD	$8.15x10^5$	$5.23x10^6$	$7.38x10^4$	UD	UD	$6.52x10^4$	$1.78x10^6$	$3.55x10^6$	$4.21x10^5$
BUN (2.4-8.2 mmol/l)	4.52	4.31	3.27	5.31	6.24	5.73	3.44	5.63	6.55	6.37	7.12	6.93
CR (31.8-116.0 μmol/l)	61.1	55.8	53.7	89.2	78.3	93.4	58.4	63.7	85.3	91.2	101.3	100.3
MELD score	ND	ND	ND	32.63	27.08	31.48	ND	ND	28.15	31.63	35.45	33.01
WBC (3.97-9.15x10^9/l)	5.22	4.73	5.87	7.12	7.55	8.23	4.63	5.82	7.05	3.87	4.05	3.83
RBC (4.09-5.74x10^{12}/l)	5.12	4.23	4.84	5.23	4.23	4.87	4.57	4.93	3.88	4.79	5.66	4.85
HB (131-172 g/l)	156	164	149	117	152	138	162	157	128	146	98	141
PLT (100-300x10^9/l)	226	214	189	211	187	156	208	195	154	205	138	213

iTRAQ, isobaric tag for relative and absolute quantification; HBV, hepatitis B virus; ACLF, acute-on-chronic liver failure; normal, normal healthy subject; patient, patients with HBV-induced ACLF; HBsAg, hepatitis B surface antigen; HBsAb, hepatitis B surface antibody; HBeAg, hepatitis B e antigen; HBeAb, hepatitis B e antibody; HBcAb, hepatitis B core antibody; HAV, hepatitis A virus; HCV, hepatitis C virus; HDV, hepatitis D virus; HEV, hepatitis E virus; AST, aspartate aminotransferase; ALT, alanine aminotransferase; TBIL, total bilirubin; PT, prothrombin time; INR, international normalized ratio; UD, undetectable; BUN, blood urea nitrogen; CR, creatinine; MELD, model for end-stage liver disease; ND, not detected; WBC, white blood cells; RBC, red blood cells; HB, hemoglobin; PLT, platelet count.

Nano LC quadrupole time-of-flight (Q-TOF) tandem mass spectrometry (MS/MS). Mass spectrometry detection was conducted using a microTOF-Q II instrument (Bruker Corp., Billerica, MA, USA), with electron spray ionization (ESI) as the ion source (cation scanning; mode, auto MS2; scan range, m/z=50-3,000). A 10-μl sample was used in the Q-TOF instrument. Following MS/MS scanning, the signal diagram was acquired. The resulting diagrams were exported to Mascot generic format (MGF) files by loading them into DataAnalysis software, version 2.1 (Bruker Corp.). Other signals were processed in a similar manner for corresponding retrieval, and the MGF documents were merged for subsequent Mascot database (Matrix Science, Ltd., London, UK) retrieval.

The resulting MS spectra were used to determine the peptide identity and abundance of each peptide in the respective spectrum. Relative abundance of a peptide was calculated by comparing the intensity of the corresponding tag.

Database searching and criteria. MicroTOF-Q control software (Bruker Corp.) was used for database searching. Following peak analysis and data processing of MS/MS signals with DataAnalysis (Bruker Corp.), the exported MGF documents were uploaded to the Mascot database for data retrieval. The search criteria were as follows: Enzyme, trypsin; database, NCBI nr_human; peptide charge, 1^+, 2^+ and 3^+; instrument, ESI-QUAD-TOF; and data format, Mascot generic. Peptide and protein identification information was thus retrieved. Mascot software was used to perform calculations based on the non-redundant protein database OWL peptide frequency (http://www.bioinf.man.ac.uk/dbbrowser/OWL/index.php) and a likelihood algorithm. The degree of confidence for protein identification was set at 95%. Injection error was corrected following automatic standardization by adjusting the software settings. Relative quantification was expressed as the average. The P-value of the degree of confidence was determined via software calculation. Hierarchical clustering analysis of the protein expression pattern was analyzed using Cluster 3.0 software. Protein annotation and classification was performed using the Database for Annotation, Visualization and Integrated Discovery functional annotation (http://david.abcc.ncifcrf.gov), selecting gene ontology biological processes, cellular components and molecular functional annotation for protein classification, and selecting the Kyoto Encyclopedia of Genes and Genomes pathway database for pathway classification and enrichment analysis.

Function identification of protein components. The functions of protein components were identified using the UniProt (http://www.uniprot.org/) and National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) databases.

Western blot analysis. Western blot analysis was performed in accordance with the instructions described in the western blotting kits (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Briefly, protein lysates were separated on a 12% polyacrylamide gel, transferred to polyvinylidene difluoride membranes and subjected to immunoblotting at 4˚C overnight with the following antibodies: Anti-keratin, type I cytoskeletal 19 (CK-19) and anti-α-1-acid glycoprotein 1 (α1-AGP) (both 1:1,000; Sigma-Aldrich, St. Louis, MO, USA), anti-carbonic anhydrase 1 (1:1,000; Abcam, Cambridge, UK) and anti-serpin peptidase inhibitor and clade A (α-1 antiproteinase, antitrypsin) member 1 (SERPINA1; 1:500; Abnova Corp., Taipei, Taiwan). After being washed, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies, and visualized using the enhanced chemiluminescence system (GE Healthcare Life Sciences, Little Chalfont, UK).

Statistical analysis. Statistical analysis was performed using SPSS software for Windows, version 13.0 (SPSS, Inc., Chicago, IL, USA). The Kolmogorov-Smimov test was performed to determine the distribution of the samples of each group. Data were expressed as the median (inter-quartile range; IIQR). A non-parametric (Kruskal-Wallis) test was applied to analyze differences between HBV-induced ACLF and normal subjects. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Protein identification. Following the Mascot MS/MS Ion Search and prior to redundancy removal, 239 proteins were identified. After redundancy removal, 124 proteins were identified, with a ≥95% degree of confidence, indicating that the results were credible. Protein quantification revealed that there were 57 proteins with ≥1.5-fold differential expression detected between the groups. Based on the score of the identified protein obtained using Cluster 3.0 software, the report threshold was 1.3 and the false positive rate of the corresponding protein was 5%. Protein quantification by software was based on the relative content of the isotopic reporter group.

Protein quantification. Protein quantification software based on the relative content of the isotopic reporter group, and using m/z=114 as a reference, showed significantly different results between the groups ($P\leq0.05$). There were 57 proteins with a ≥1.5-fold difference between the HBV-induced ACLF patients and the normal patients, as analyzed by iTRAQ (Table II). Among the 57 proteins, 10 categories of proteins were classified based on their function: Regulatory molecule, protease, transporter, structure protein, hydrolase, calcium binding protein, receptor, signaling molecule, extracellular matrix and unclassified (Fig. 1).

Following a preliminary selection of the 57 proteins, 4 proteins with the most marked differences in expression and the most significant association with liver diseases (Table II) were selected to be verified by western blot analysis. These proteins were CK-19, α1-AGP, carbonic anhydrase 1 and SERPINA1. After the verification of 6 hepatic tissue samples by western blot analysis, the results were nearly identical to the results of the iTRAQ analyses (Figs. 2-5).

Discussion

In the field of CHB research, proteomics is not widely performed due to the complexity of the pathogenesis of CHB. In the present study, a western blot-verified iTRAQ approach was used to quantify the differences in specific protein expression by comparing liver tissue samples from healthy individuals and HBV-induced ACLF patients. The results showed differ-

Table II. Results of the iTRAQ test.

No.	Identified protein name	Accession number	Molecular weight, kDa	Biological processes	Molecular function	Protein function	Protein expression in ACLF patient liver tissue (fold change)
1	UDP glucuronosyltransferase 2 family, polypeptide B7, isoform CRA_b	Unclassified	60	Unclassified	Unclassified	Unclassified	Downregulation (3.031)
2	Hydroxyacid oxidase 1	Q9UJM8	41	Fatty acid α-oxidation	Oxidoreductase	Protease	Downregulation (2.000)
3	Membrane-associated progesterone receptor component 1	O00264	22	Unclassified	Receptor	Receptor	Downregulation (2.000)
4	Enyol-CoA: hydratase/3-hydroxyacyl-CoA dehydrogenase	Unclassified	79	Unclassified	Unclassified	Unclassified	Downregulation (1.866)
5	Soluble epoxide hydrolase	P07099	63	Aromatic hydrocarbon catabolism, detoxification	Hydrolase	Hydrolase	Downregulation (1.866)
6	Carboxylesterase	P23141	61	Response to toxin	Hydrolase, serine esterase	Hydrolase	Downregulation (1.741)
7	Catalase	P04040	60	Hydrogen peroxide	Mitogen, oxidoreductase, Peroxidase	Protease	Downregulation (1.625)
8	ACSL1 protein	P33121	60	Fatty acid and lipid metabolism	Ligase	Protease	Downregulation (1.625)
9	3-Ketoacyl-CoA thiolase, peroxisomal isoform a	P09110	44	Fatty acid and lipid metabolism	Acyltransferase, transferase	Protease	Downregulation (1.625)
10	Epoxide hydrolase 1	P07099	53	Aromatic compound catabolic process, response to toxin	Hydrolase	Hydrolase	Downregulation (1.625)
11	4-Hydroxyphenylpyruvate-dioxygenase	P32754	45	Phenylalanine catabolism, Tyrosine catabolism	Dioxygenase, Oxidoreductase	Protease	Downregulation (1.625)
12	Acyl-CoA thioesterase 1	Q86TX2	46	Acyl-CoA metabolic process	Hydrolase, serine esterase	Hydrolase	Downregulation (1.625)
13	Galactokinase 1	B4E1G6	45	Unclassified	Kinase, transferase	Protease	Downregulation (1.625)
14	Neuroendocrine specific protein c homolog	Unclassified	22	Unclassified	Unclassified	Unclassified	Downregulation (1.625)
15	Hepatic peroxysomal alanine: glyoxylate aminotransferase	Q9BXA1	40	Unclassified	Aminotransferase, transferase	Protease	Downregulation (1.516)
16	Betaine-homocysteine methyltransferase	Q93088	45	Amino-acid betaine catabolic process, cellular nitrogen compound metabolic process	Methyltransferase, transferase	Protease	Downregulation (1.516)
17	Glutamate dehydrogenase 1, mitochondrial precursor	P00367	61	Glutamate biosynthetic process	Oxidoreductase	Protease	Downregulation (1.516)
18	D-dopachrome decarboxylase	P30046	13	Melanin biosynthesis	Lyase, D-dopachrome decarboxylase activity	Protease	Downregulation (1.516)
19	Nicotinate phosphoribosyltransferase domain containing 1, isoform CRA_c	C9J8U2	56	NAD biosynthetic process	Glycosyltransferase, transferase	Protease	Downregulation (1.516)
20	Adenylate kinase 2, isoform CRA_c	P54819	18	Nucleobase-containing small molecule interconversion	Kinase, transferase	Protease	Downregulation (1.516)
21	Argininosuccinate synthetase, isoform CRA_b	P00966	51	Acute-phase response	ATP binding, argininosuccinate synthase activity	Protease	Upregulation (1.516)
22	Tropomyosin β chain isoform 2	P07951	33	Muscle contraction, regulation	Muscle protein	Structure	Upregulation (1.516)

Table II. Continued.

No.	Identified protein name	Accession number	Molecular weight, kDa	Biological processes	Molecular function	Protein function	Protein expression in ACLF patient liver tissue (fold change)
				of ATPase activity		protein	
23	Annexin A2, isoform CRA_c	P07355	32	Positive regulation of vesicle fusion	Calcium ion binding	Calcium binding protein	Upregulation (1.516)
24	Transferrin	Q06AH7	77	Cellular iron ion homeostasis, iron ion transport	Ferric iron binding	Transporter	Upregulation (1.516)
25	Histone H2B type 1-D	P58876	14	Nucleosome assembly	DNA binding	Protease	Upregulation (1.516)
26	Lumican precursor	P51884	38	Carbohydrate metabolic process	Extracellular matrix structural constituent	Structure protein	Upregulation (1.516)
27	YWHAZ protein	P63104	35	Anti-apoptosis, signal transduction	Protein domain specific binding, transcription factor binding	Signaling molecule	Upregulation (1.516)
28	L-lactate dehydrogenase A chain isoform 1	P00338	37	Glycolysis	Oxidoreductase	Protease	Upregulation (1.625)
29	Phosphoglycerate kinase 1	P00558	45	Glycolysis	ATP binding, phosphoglycerate kinase activity	Protease	Upregulation (1.625)
30	α-2-macroglobulin precursor	P01023	163	Blood coagulation, intrinsic pathway	Serine-type endopeptidase inhibitor activity	Protease	Upregulation (1.625)
31	Manganese-containing superoxide dismutase	Unclassified	24	Unclassified	Unclassified	Unclassified	Upregulation (1.625)
32	T-plastin polypeptide	Unclassified	64	Unclassified	Unclassified	Unclassified	Upregulation (1.625)
33	Peroxiredoxin 2, isoform CRA_a	A6NIW5	15	Unclassified	Antioxidant activity, oxidoreductase activity	Protease	Upregulation (1.625)
34	Filamin-A isoform 1	P21333	280	Actin crosslink formation and actin cytoskeleton reorganization	Binding	Regulatory molecule	Upregulation (1.625)
35	Annexin A1, isoform CRA_b	P04083	40	Anti-apoptosis, cell surface receptor linked signaling pathway	Phospholipase A2 inhibitor, calcium ion binding	Calcium binding protein	Upregulation (1.625)
36	Polyubiquitin	P0CG47	68	DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest	DNA repair	Regulatory molecule	Upregulation (1.741)
37	Apolipoprotein J precursor	Unclassified	49	Unclassified	Unclassified	Unclassified	Upregulation (1.741)
38	Hemopexin precursor	Unclassified	52	Unclassified	Unclassified	Unclassified	Upregulation (1.741)
39	Pyruvate kinase, muscle, isoform CRA_b	P30613	38	Glycolysis	ATP binding, magnesium ion binding	Protease	Upregulation (1.866)
40	Annexin A5, isoform CRA_c	P08758	33	Blood coagulation, hemostasis	Calcium ion binding	Calcium binding protein	Upregulation (2.000)
41	Keratin 1 [*Homo sapiens*]	Q16195	66	Unclassified	Structural molecule activity	Structure protein	Upregulation (2.000)
42	Glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1), isoform CRA_a	Unclassified	32	Unclassified	Unclassified	Unclassified	Upregulation (2.144)
43	Myosin, heavy polypeptide 11,	D2JYH7	226	Unclassified	Motor protein	Transporter	Upregulation (2.297)

Table II. Continued.

No.	Identified protein name	Accession number	Molecular weight, kDa	Biological processes	Molecular function	Protein function	Protein expression in ACLF patient liver tissue (fold change)
	smooth muscle, isoform CRA_c						
44	Collagen α-1 (XIV) chain precursor	Q05707	194	Cell adhesion	Extracellular matrix structural constituent	Extracellular matrix	Upregulation (2.297)
45	Nicotinamide N-methyltransferase	P40261	30	Organ regeneration	Nicotinamide N-methyltransferase activity	Protease	Upregulation (2.297)
46	Osteoglycin	Q7Z532	34	Unclassified	Protein binding	Extracellular matrix	Upregulation (2.297)
47	Unnamed protein product	Unclassified	52	Unclassified	Unclassified	Unclassified	Upregulation (2.639)
48	Apolipoprotein A-I, isoform CRA_b	P02647	23	Cholesterol metabolism, transport	Beta-amyloid binding, cholesterol binding, cholesterol transporter activity	Transporter	Upregulation (2.639)
49	Fibrinogen α chain isoform α preproprotein	P02671	70	Platelet activation, protein polymerization, response to calcium ion, signal transduction	Eukaryotic cell surface binding, protein binding, bridging, receptor binding	Extracellular matrix	Upregulation (2.639)
50	Unnamed protein product	Unclassified	16	Unclassified	Unclassified	Unclassified	Upregulation (2.828)
51	JC-κ protein - human	Unclassified	15	Unclassified	Unclassified	Unclassified	Upregulation (3.031)
52	Albumin, isoform CRA_h	P02768	69	Transport	Binding capacity	Transporter	Upregulation (3.482)
53	Hemoglobin α 1 globin chain	Q9BX83	11	Transport	Heme binding, oxygen binding, oxygen transporter activity	Transporter	Upregulation (4.595)
54	Keratin, type I cytoskeletal 19	P08727	44	Host-virus interaction	Structural constituent of cytoskeleton and muscle	Structure protein	Upregulation (3.732)
55	α-1-acid glycoprotein 1	P02763	24	Acute-phase response, regulation of immune system process	Protein binding	Regulatory molecule	Upregulation (4.595)
56	Carbonic anhydrase 1	P00915	29	One-carbon metabolic process	Carbonate dehydratase activity, zinc ion binding	Protease	Upregulation (3.031)
57	Serpin peptidase inhibitor, clade A (α-1 antiproteinase, antitrypsin), member 1	P01009	47	Unclassified	Serine-type endopeptidase inhibitor activity	Regulatory molecule	Upregulation (2.297)

A total of 57 proteins were found to exhibit a ≥1.5-fold difference in expression levels between the liver tissue of patients with hepatitis B virus-induced ACLF and that of normal subjects. Four proteins (nos. 54-57) with the largest differences in their expression and the most significant association with liver disease were selected to be verified by western blot assay, using 6 hepatic tissue samples. Normal subjects expressed normal levels of all the proteins listed. iTRAQ, isobaric tag for relative and absolute quantification; ACLF, acute-on-chronic life failure; NAD, nicotinamide adenine dinucleotide; ATP, adenosine triphosphate.

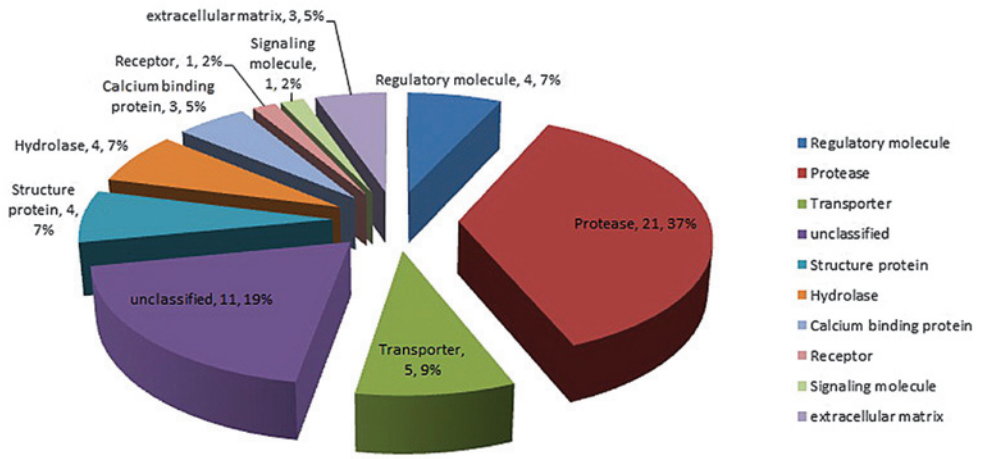

Figure 1. Classification of the 57 proteins in different categories based on protein function. A total of 10 protein function categories were detected: Regulatory molecule, protease, transporter, structure protein, hydrolase, calcium-binding protein, receptor, signaling molecule, extracellular matrix and unclassified.

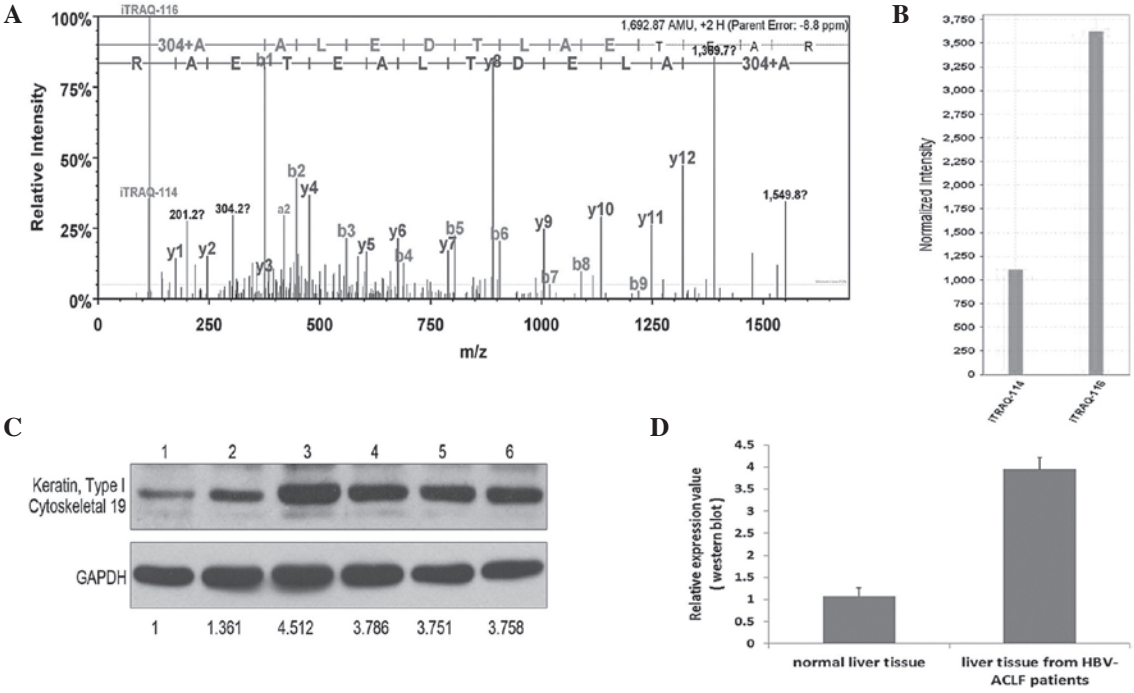

Figure 2. Detection results of keratin, type I cytoskeletal 19 (peptide sequence AALEDTLAETEAR). (A) Mass spectrum of the peptide with the sequence 'AALEDTLAETEAR'. Fragment ions assigned to y-ions and b-ions are labeled. (B) iTRAQ mass spectrometry results show that the expression of 116 was considerably higher than that of 114 (labeling reagent 114 for normal liver tissue, labeling reagent 116 for liver tissue from patients with HBV-induced ACLF). (C) Results of western blot verification: Lanes 1 and 2, normal liver tissue; lanes 3-6, liver tissue from patients with HBV-induced ACLF. (D) Relative expression value of the western blotting. iTRAQ, isobaric tag for relative and absolute quantification; ACLF, acute-on-chronic liver failure; HBV, hepatitis B virus.

ences in specific proteins between the two groups, which may elucidate the deregulated pathways and networks involved in the proteomic mechanism underlying this disease. The 4 proteins with the greatest differences in expression between groups and the most significant association with liver diseases were selected to be verified by western blot analysis. These proteins included 1 structural protein, 2 regulatory proteins and 1 protease (Table II). The aim was to determine if these proteins were involved in the pathogenesis of HBV-induced ACLF.

CK-19, a member of the keratin family, can be found in a defined zone of basal keratinocytes, sweat glands, mammary gland ductal and secretory cells, bile ducts, the gastrointestinal tract, the bladder urothelium, oral epithelia, the esophagus and the ectocervical epithelium. The expression levels of CK-19 can be used as a luminal epithelial cell marker expressed in the majority of breast carcinomas and not typically detected in lymph nodes (12). Furthermore, CK-19 has been reported to be a novel prognostic factor in non-small-cell lung cancer (13); however, the exact function of CK-19 in the pathogenesis of HBV-induced liver failure remains unclear and requires verification by cell function studies involving larger sample sizes.

α1-AGP is a major acute-phase protein that is synthesized in the hepatocytes of humans, rats, mice and other species. An increase in the serum concentration of α1-AGP may occur as a response to systemic tissue injury, inflammation or infection

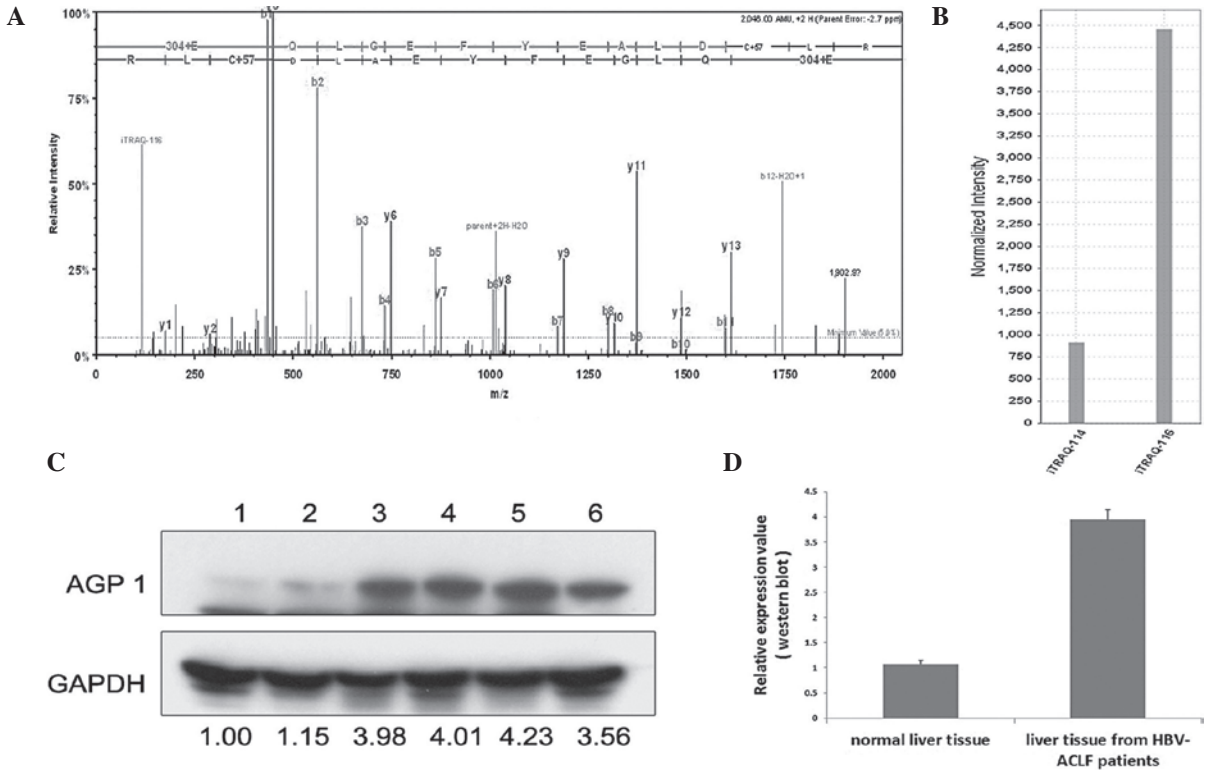

Figure 3. Detection results of AGP 1 (peptide sequence EQLGEFYEALDCLR). (A) Mass spectrum of the peptide 'EQLGEFYEALDCLR'. Fragment ions assigned to y-ions and b-ions are labeled. (B) iTRAQ mass spectrometry results show that the expression of 116 was considerably higher than that of 114 (labeling reagent 114 for normal liver tissue, labeling reagent 116 for liver tissue from patients with HBV-induced ACLF). (C) Results of the western blot verification: Lanes 1 and 2, normal liver tissue; lanes 3-6, liver tissue from patients with HBV-induced ACLF. (D) Relative expression value of the western blotting. iTRAQ, isobaric tag for relative and absolute quantification; AGP1, α-1-acid glycoprotein 1; HBV, hepatitis B virus; ACLF, acute-on-chronic liver failure.

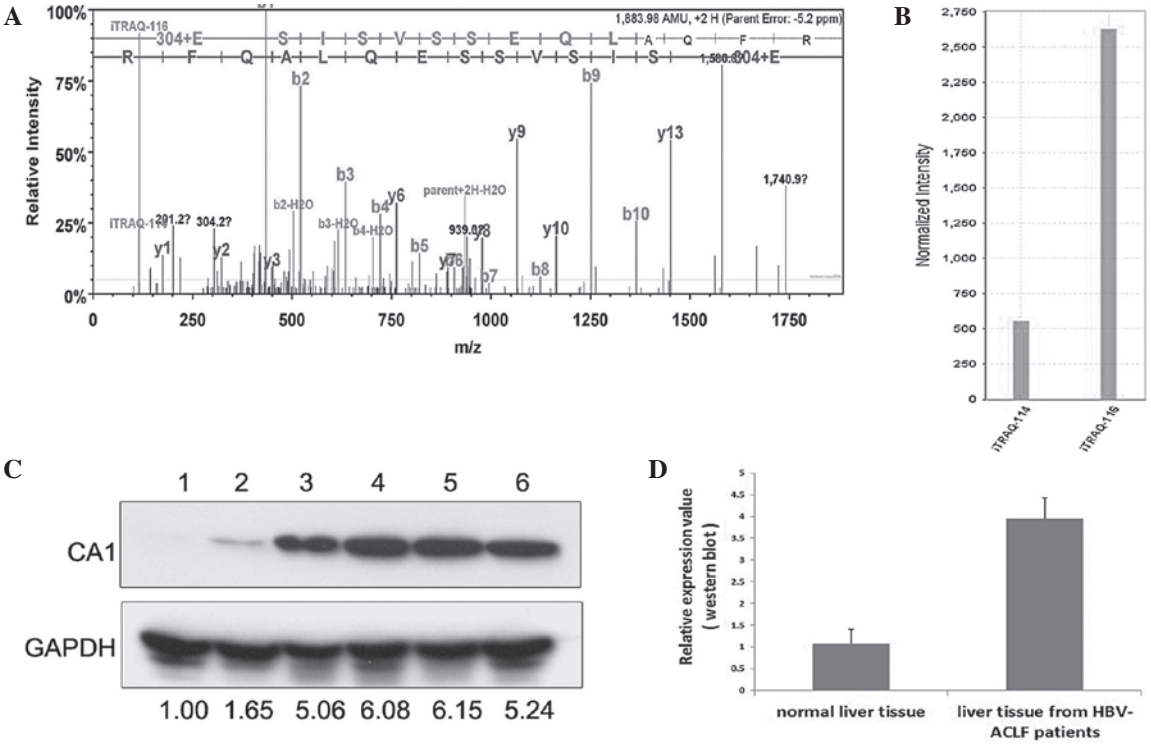

Figure 4. Detection results of CA1 (peptide sequence ESISVSSEQLAQFR). (A) Mass spectrum of the peptide 'ESISVSSEQLAQFR'. Fragment ions assigned to y-ions and b-ions are labeled. (B) iTRAQ mass sspectrometry results show that the expression level of 116 was considerably higher than that of 114 (labeling reagent 114 for normal liver tissue, labeling reagent 116 for liver tissue from patients with HBV-induced ACLF). (C) Results of the western blot verification: Lanes 1 and 2, normal liver tissue; lanes 3-6, liver tissue from patients with HBV-induced ACLF. (D) Relative expression value of the western blotting. iTRAQ, isobaric tag for relative and absolute quantification; CA1, carbonic anhydrase 1; HBV, hepatitis B virus; ACLF, acute-on-chronic liver failure.

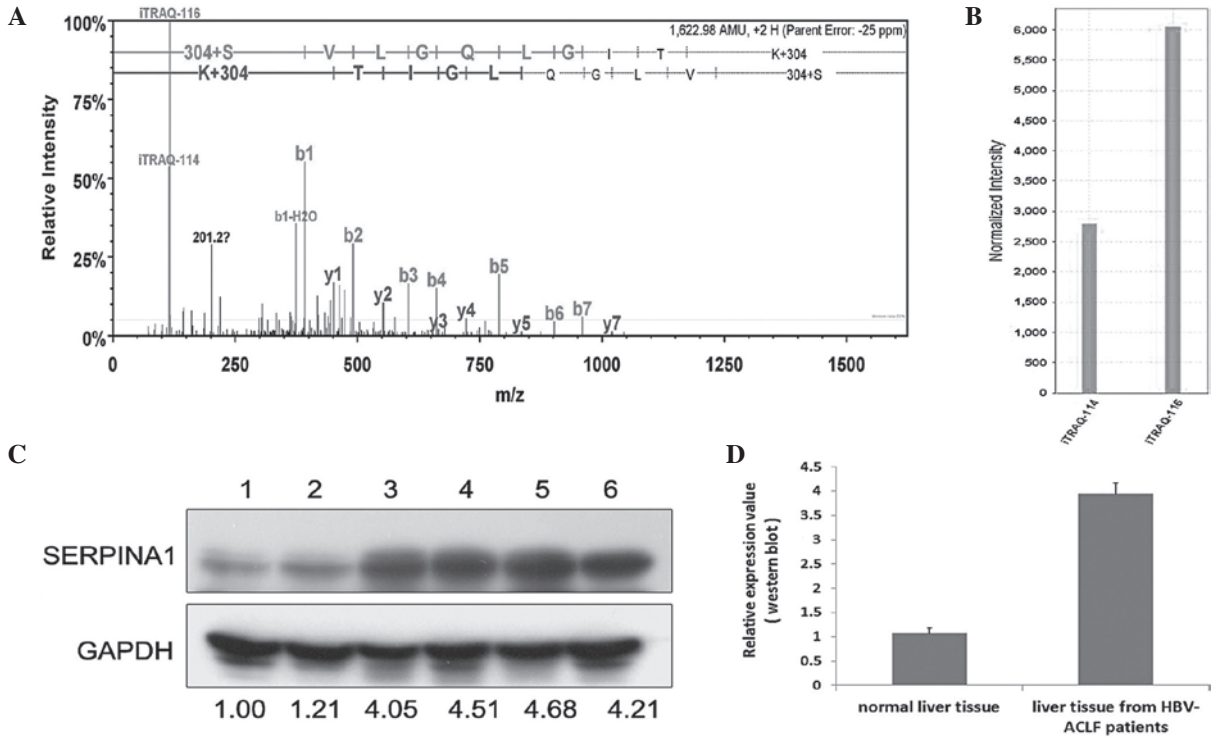

Figure 5. Detection results of SERPINA1 (peptide sequence SVLGQLGITK). (A) Mass spectrum of the peptide 'SVLGQLGITK'. Fragment ions assigned to y-ions and b-ions are labeled. (B) iTRAQ mass spectrometry results show that expression of 116 was considerably higher than that of 114 (labeling reagent 114 for normal liver tissue, labeling reagent 116 for liver tissue from patients with HBV-induced ACLF). (C) Results of the western blot verification: Lanes 1 and 2, normal liver tissue; lanes 3-6, liver tissue from patients with HBV-induced ACLF. (D) Relative expression value of the western blotting. iTRAQ, isobaric tag for relative and absolute quantification; SERPINA1, serpin peptidase inhibitor and clade A (α-1-antiproteinase, antitrypsin) member 1; HBV, hepatitis B virus; ACLF, acute-on-chronic liver failure.

and is considered to be associated with an enhanced rate of hepatic synthesis. The biological function of α1-AGP remains unknown, although a variety of its immunomodulating effects and functions have been described (14). In addition, it has been reported that α1-AGP levels are increased in gastric tissue and in the plasma of patients with carcinoma of the stomach (15). Ren *et al* (3) reported that α1-AGP may be a potential biomarker for ACLF induced by CHB. Their results showed that α1-AGP levels decreased significantly in the plasma of patients with HBV-induced ACLF, but decreased to a lesser extent in the plasma of patients with CHB. By contrast, the results of the present study showed that α1-AGP expression was 4.595-fold greater in the HBV-induced ACLF liver tissue than that in the liver tissue of healthy subjects, giving rise to the theory that α1-AGP may have collected in the liver from the blood in response to the process of ACLF. Further cell experiments are required to validate the function of α1-AGP in the reaction and to analyze the levels in the blood and liver tissue of the same patient.

Carbonic anhydrase is among the most important protein components of erythrocytes. The primary functions of carbonic anhydrase are to modulate acid-base balance in the blood and other tissues, assist in the removal of CO_2 and ensure a moderate substrate concentration for enzymes using CO_2 and HCO_3 as substrates. Numerous members of the carbonic anhydrase family exist. One member, carbonic anhydrase isoenzyme 9, is abnormally expressed in gastrointestinal and gynecological tumors. Carbonic anhydrase 9 has potential clinical value, including as a biomarker of human colonic mucinous carcinoma and increased proliferation in the colorectal mucosa (16-18). Additionally, it has been demonstrated that carbonic anhydrase 3 levels are significantly reduced in the livers of superoxide dismutase-deficient mice, although immunohistochemical analysis revealed that the reduction was not homogenous throughout the lobular structure of the liver (19). The function of carbonic anhydrase 1 in HBV-induced ACLF, however, is not clear. The results of the present study showed that its expression increased 3-fold in patients with HBV-induced ACLF. The specific role and function of carbonic anhydrase 1 requires further verification.

Previous studies have investigated SERPINA1 in liver disease. In hepatitis C, following Basic Local Alignment Search Tool analysis in the positive clones, 3 proteins that interacted with the HCV NS3 protease were serpin peptidase inhibitor, clade A, member 1, and cyclophilin-LC (20). α-1-antiproteinase has potential use as a biomarker for the non-invasive diagnosis of liver fibrosis, which may benefit the follow-up of HCV patients (21). Furthermore, α-1-antiproteinase deficiency is an autosomal recessive disorder in HCC that results from point mutations in the SERPINA1 gene, which are associated with neonatal hepatitis and cirrhosis, as well as HCC (22). α-1-antiproteinase may also be a biomarker (protein marker) for HCC (23) and have potential as a biomarker for diagnosing CHB, as Tan *et al* (24) reported that the protein was highly expressed in serum samples from HCC patients and severe chronic hepatitis patients. Furthermore, α-1-antiproteinase is expressed most markedly

in normal tissue and cells and exhibits reduced expression in tissues and cells from HCC patients and severe chronic hepatitis patients, which indicates the specific secretion of α-1-antiproteinase from tissues and cells into the serum (24). The present results, however, demonstrated that SERPINA1 expression was increased 2.297-fold in liver tissue samples from patients with HBV-induced ACLF. This may have been due to the fact that not all of the patients in the present study exhibited cirrhosis, meaning that they were in a less-advanced disease course. Thus, the majority of the SERPINA1 had not been secreted from the liver into the serum. Further studies with larger sample sizes and cell function experiments are therefore required.

In conclusion, the biological function of these proteins in HBV-induced ACLF remains unclear, and it is difficult to determine whether or not the differences in protein level were the result or the origin of the ACLF. Additionally, certain results obtained in the present study are inconsistent with those of previous studies. Further functional studies are required, including studies using a larger sample and cell function experiments. The proteins showing differential expression that have been described in the present study may not be suitable for use as biomarkers for the clinical prognostic index of CHB, as this is an invasive method; however, by identifying specific proteins and protein derangements, further insight may be obtained into the deregulated pathways and networks involved in the pathogenesis of HBV-induced ACLF. Furthermore, the present proteomic study may be useful and valuable for future studies of the protein mechanisms underlying the pathogenesis of CHB.

Acknowledgements

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