Research Article

iTRAQ-Based Proteomics Identification of Serum Biomarkers of Two Chronic Hepatitis B Subtypes Diagnosed by Traditional Chinese Medicine

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Received 18 July 2016; Accepted 24 October 2016

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Background. Chronic infection with hepatitis B virus (HBV) is a leading cause of cirrhosis and hepatocellular carcinoma. By traditional Chinese medicine (TCM) pattern classification, damp heat stasis in the middle-jiao (DHSM) and liver Qi stagnation and spleen deficiency (LSSD) are two most common subtypes of CHB. Results. In this study, we employed iTRAQ proteomics technology to identify potential serum protein biomarkers in 30 LSSD-CHB and 30 DHSM-CHB patients. Of the total 842 detected proteins, 273 and 345 were differentially expressed in LSSD-CHB and DHSM-CHB patients compared to healthy controls, respectively. LSSD-CHB and DHSM-CHB shared 142 upregulated and 84 downregulated proteins, of which several proteins have been reported to be candidate biomarkers, including immunoglobulin (Ig) related proteins, complement components, apolipoproteins, heat shock proteins, insulin-like growth factor binding protein, and alpha-2-macroglobulin. In addition, we identified that proteins might be potential biomarkers to distinguish LSSD-CHB from DHSM-CHB, such as A0A0A0MS51_HUMAN (gelsolin), PON3_HUMAN, Q96K68_HUMAN, and TRPM8_HUMAN that were differentially expressed exclusively in LSSD-CHB patients and A0A087WT59_HUMAN (transthyretin), ITIH1_HUMAN, TSP1_HUMAN, CO5_HUMAN, and ALBU_HUMAN that were differentially expressed specifically in DHSM-CHB patients. Conclusion. This is the first time to report serum proteins in CHB subtype patients. Our findings provide potential biomarkers can be used for LSSD-CHB and DHSM-CHB.

1. Introduction

Chronic hepatitis B virus (CHB) infection is a leading cause of cirrhosis and hepatocellular carcinoma (HCC) and, in addition to morbidity and mortality, creates significant economic and social burdens [1, 2]. It is estimated that approximately 240 million people have CHB infection worldwide and CHB infection should be responsible for 650,000 cases of hepatocellular carcinoma [2, 3]. Due to the pathogenicity of CHB, early detection of CHB infection is the goal of treatment to diagnose and prevent the progression [4]. To this end, several hepatitis B virus (HBV) markers have been identified, including antigens (hepatitis B surface antigen, HBsAg; hepatitis B e antigen, HBeAg; hepatitis B core antigen, HBcAg), antibodies (hepatitis B surface antibody, anti-HBs; hepatitis B e antibody, anti-HBe; hepatitis B core antibody, anti-HBc), and immunoglobulin (Ig) G and immunoglobulin M; however, unequivocal diagnosis requires more biomarkers [5].
By traditional Chinese medicine (TCM) pattern classification, CHB infected patients are accordingly classified into six subtypes [6]: (1) damp heat stasis in the middle-jiao (DHSM), (2) liver Qi stagnation and spleen deficiency (LSSD), (3) Yang deficiency of spleen and kidney (YDSK), (4) Yin deficiency of liver and kidney (YDCLK), (5) blood stasis into collateral (BSIC), and (6) damp heat complicated with blood stasis (DHBS). Among them DHSM and LSSD are two most common CHB subtypes and have unique syndromes in clinic. For example, LSSD patients always have main syndromes, such as (Mi) flank pain and (Mii) abdominal distension and loose stools, and secondary symptoms, including (Si) depression and boredom, (Sii) body tired fatigue, and (Siii) pale tongue with teeth marks. DHSM patients have another two main syndromes, such as (Mi) abdominal distension and (M2) yellow greasy moss, and three secondary syndromes, including (Si) nausea, being tired of the oil, and poor appetite, (S2) jaundice, bright color, and dark urine, and (S3) viscous stool foul smell. However, these syndromes are diagnosed by TCM doctors according to their experiences and the molecular biomarkers remain unclear.

Proteomics is a powerful technology recently developed to enhance our study on the diagnosis, treatment, and prevention of human diseases [7]. Among the proteomics technologies iTRAQ (isobaric Tags for Relative and Absolute Quantitation) has become popular for protein identification and quantification due to its sensitivity, accuracy, and high throughput [8]. It has been used to identify biomarker proteins for different stages of hepatitis B related diseases in patients and cellular models [9–12]. Several serum proteins have been reported to be potential biomarkers for CHB, such as actin [13], apolipoproteins A-I and A-IV [14], complement component [15], immunoglobulin related proteins [15, 16], haptoglobins β and α2 chain [14], and transferrin [17].

In this study, we employed iTRAQ combined with LC-ESI-MS/MS analyses to investigate protein biomarkers in the serum samples of two CHB subtype patients (LSSD and DHSM). Compared to healthy controls we found a number of proteins differentially expressed in both LSSD and DHSM CHB subtypes, such as actin, apolipoprotein, complement component, and immunoglobulin related proteins. In addition, we identified some proteins differentially expressed exclusively in one of LSSD and DHSM groups, such as gelsolin (GSN), likely SNC73 protein, and transient receptor potential cation channel subfamily M member 8 (TRPM8) that were found with different expression in LSSD-CHB patients only and transthyretin (TTR), tubulin, and keratin types I and II that were differentially expressed in DHSM-CHB patients only. Our findings not only validate previously reported CHB protein biomarkers but also report for the first time protein biomarkers for LSSD and DHSM CHB subtypes. The output of this study gives a valuable resource for future HBV associated studies and provides new insights of traditional Chinese medicine on molecular level.

2. Materials and Methods

2.1. Ethics Statement. This study was conducted in compliance with the Declaration of Helsinki, the ethics approval was granted by the research medical ethics committee of Chengdu University of Traditional Chinese Medicine, and signed informed consent was obtained from all participants.

2.2. Patients and Serum Collection. A total of 104 CHB patients were enrolled from West China Hospital, Sichuan University, and filtered with strict clinical evaluation described below. For iTRAQ proteomics analysis, we obtained blood samples from 30 LSSD-CHB patients, 30 DHSM-CHB patients, and 20 healthy controls (HCTL). For western blot analysis, 9 LSSD-CHB patients, 9 DHSM-CHB patients, and 6 HCTL participants were enrolled. Serum was collected from blood sample (4 mL) following the manufacture’s protocol. Briefly, blood sample was incubated at room temperature for 2 h in vacutainer blood handling tube (Becton Dickinson, New Jersey, USA) and centrifuged for 10 min at 3,000 rpm and 4°C. Serum sample, which is the result supernatant, was transferred into a clean polypropylene tube and stored at −80°C.

2.3. Clinical Evaluation. The viral markers HBsAg, HBeAg, anti-HBs, anti-HBc, and anti-HBe were determined routinely in serum samples using standard procedures (AxSYM®, Abbott Laboratories, Rungis, France), as well as other molecular diagnostic markers like ALT (alanine transaminase), AST (aspartate aminotransferase), STB (serum total bilirubin), CB (conjugated bilirubin), UCB (unconjugated bilirubin), and HBV-DNA. Participants, who have hepatitis B history or HBsAg positive history for more than six months, were diagnosed as chronic HBV infection if they were positive to HBsAg and/or HBV-DNA. We used both western and Chinese medicine criteria to divide CHB patients into two groups. First, participants were satisfied with the following requirements: (1) serum HBsAg positive for over 6 months; (2) HBV-DNA positive; (3) continuous or repeated elevated serum ALT in last 12 months; (4) being 18–60 years old; (5) no planned move during the test. Then, LSSD-CHB and DHSM-CHB patients were diagnosed using the clinical symptoms mentioned before. CHB patients were diagnosed as LSSD-CHB when they met the criteria: (1) Mi and Mii; (2) Mi, Sii, and Siii; (3) Mii and Si. DHSM-CHB patients were diagnosed as follows: (1) M1 and M2; (2) M1, S1, and S2; (3) M2 and two of the secondary symptoms. We also filtered the patients when they satisfied one of the following criteria: (1) being associated with other types of hepatitis viruses or human immunodeficiency virus (HIV); (2) cirrhosis, malignancy; (3) being diagnosed with fulminant hepatitis (including acute, subacute, and chronic severe hepatitis); (4) being associated with drug or toxic liver, autoimmune hepatitis, and genetic-metabolic liver disease; (5) heart, lung, kidney, endocrine, blood, and other serious diseases; (6) pregnant women and lactating women; (7) mental disorders, in line with Chinese Classification of Mental Disorders Diagnosis (CCMD-3) standard; (8) other individuals not suitable for the cohort study.

2.4. Protein Preparation. Serum sample (200 μL) from each patient was processed to reduce the complexity by using
ProteoMiner™ Kits (Bio-Rad Laboratories, Hercules, CA, USA). Then, the sample was eluted using Lysis buffer at pH 8.5 (2 M Thiourea, 7 M Urea, 4% CHAPS, and 40 mM Tris-HCl), reduced using 10 mM DTT at 56°C for 1 h, and alkylated using 55 mM IAM in darkness for 1 h. After being precipitated within chilled acetone (4 x volume) at −20°C overnight, the protein sample was centrifuged at 30,000 x g for 15 min at 4°C; the pellet was next dissolved in 500 μL of 0.5 M triethylammonium bicarbonate (Applied Biosystems, Milan, Italy) and sonicated at 200 W in ice for 15 min. Finally, the samples were centrifuged again at 30,000 x g for 15 min at 4°C, and the supernatant was quantified using Bradford Protein Assay Kit (CWBio, Beijing, China) and stored at −80°C for subsequent analysis.

2.5. iTRAQ Sample Labelling, SCX Fractionation, and LC-ESI-MS/MS Analysis. Proteins isolated from 10 individuals were pooled for iTRAQ labelling. Pooled protein samples (100 μg; variable modifications: dioxidation (M), oxidation (M), carboxymethyl (C), iTRAQ8plex (N-term) and iTRAQ8plex (Y), mass values: monoisotopic; peptide mass tolerance: ±15 ppm; fragment mass tolerance: ±20 mmu; max missed cleavages: (1) The charge states of peptides were set to +2 and +3. Specifically, an automatic decoy database search was performed in Mascot by choosing the decoy checkbox in which a random sequence of database is generated and tested for raw spectra as well as the real database. To reduce the probability of false peptide identification, only peptides at the 95% confidence interval by a Mascot probability analysis greater than “identity” were counted as identified. And each confident protein identification involves at least one unique peptide.

2.7. Protein Different Expression and Functional Analysis. To identify differentially expressed proteins in LSSD-CHB and DHSM-CHB compared to HCTL, we set a cut-off for fold change (>1.2) of protein abundance provided by Mascot and p value (<0.05) calculated by edgeR [22]. Venn diagram of up- and downregulated proteins was analyzed by InterActiVenn (http://www.interaktivenn.net/) [23]. To annotate potential functions of proteins, UniProt IDs of candidate proteins were submitted to DAVID Bioinformatics Resources 6.7 (https://david.ncifcrf.gov/home.jsp) [24] and STRING v10 (http://string-db.org/) [25], Gene Ontology (GO), and KEGG pathway were selected, and we used false discovery rate (FDR) to control the results. Protein-protein interaction networks were analyzed by STRING.

2.8. Western Blot Analysis. Protein samples obtained from serum of 9 LSSD-CHB patients, 9 DHSM-CHB patients, and 6 healthy individuals were resolved by 12% SDS-PAGE using MiniProtein II electrophoresis unit (Bio-Rad) run at constant 120 V for 1 h and transferred to a PVDF membrane (Amersham Biosciences) under a constant voltage of 15 V for 20 min. The membranes were blocked with 5% skim milk powder in Tris-buffered saline with 0.05% Tween-20 (TTBS) for 1 h and probed in TTBS with primary antibodies (1:500, Santa Cruz Biotechnology, CA, USA), anti-PSMA7 (sc-166761), anti-PF4V (sc-367359), anti-PSMA6 (sc-271187), anti-SERPING1 (sc-377062), anti-ACTB (sc-8432), anti-AHSG (sc-137102), anti-CTSC (sc-74590), anti-PLTP (sc-271596), and anti-ALB (sc-46293), followed by incubation with secondary antibody (1:1000) for 1 h in darkness. All antibody incubations were carried out using gentle orbital shaking at room temperature. Western blots were washed five times in TTBS (5 min x 2 and 10 min x 3) after each incubation step and visualized with enhanced chemiluminescence (ECL, GE Healthcare) following the manufacturers’ instructions. Band intensities on the Western blots were quantified using ImageJ (Wayne Rasband, National Institutes of Health). Albumin was used as reference to calculate the relative intensity of each protein. Then, mean ± SD values of each protein in HCTL and patients were calculated and compared using GraphPad Prism (http://www.graphpad.com/).

2.9. Statistical Analysis. Statistical analysis including the calculation of mean value, standard deviation (SD), and students’ t-test was performed by using GraphPad Prism (v
Table 1: Clinical diagnosis of patients who participated in this study.

| Diagnosis                                      | Unit | LSSD-CHB (n = 30) | DHSM-CHB (n = 30) | HCTL (n = 20) | p value |
|------------------------------------------------|------|-------------------|-------------------|---------------|---------|
| Sex                                            |      |                   |                   |               |         |
| Male                                           |      | 24                | 15                | 10            |         |
| Female                                         |      | 6                 | 15                | 10            |         |
| Age                                            | Years| 17−56             | 18−60             | 24−56         |         |
| Mean age                                       |      | 30.8              | 36.83             | 36.15         |         |
| Standard deviation (SD)                        |      | 10.526            | 11.885            | 11.554        |         |
| Hepatitis B surface antigen (HBsAg)            |      |                   |                   |               |         |
| Positive                                       |      | 28                | 29                |               |         |
| Negative                                       |      | 0                 | 0                 |               |         |
| Hepatitis surface antibody (anti-HBs)          |      |                   |                   |               |         |
| Positive                                       |      | 2                 | 0                 |               |         |
| Negative                                       |      | 25                | 29                |               |         |
| Hepatitis Be antigen (HBeAg)                   |      |                   |                   |               |         |
| Positive                                       |      | 21                | 19                |               |         |
| Negative                                       |      | 7                 | 9                 |               |         |
| Hepatitis Be antibody (anti-HBe)               |      |                   |                   |               |         |
| Positive                                       |      | 9                 | 12                |               |         |
| Negative                                       |      | 19                | 16                |               |         |
| Hepatitis B core antibody (anti-HBc)           |      |                   |                   |               |         |
| Positive                                       |      | 28                | 29                |               |         |
| Negative                                       |      | 0                 | 0                 |               |         |
| Alanine transaminase (ALT)                     | IU/L | 13.8−627          | 34−673            | 1.0           |
| Mean ALT level                                  |      | 190.153           | 187.393           |               |         |
| SD                                             |      | 161.231           | 177.618           |               |         |
| Aspartate aminotransferase (AST)               | IU/L | 26.5−345          | 28−556            | 0.9999        |
| Mean AST                                       |      | 96.74             | 142.427           |               |         |
| SD                                             |      | 71.699            | 153.539           |               |         |
| Serum total bilirubin (STB)                    | umol/L| 10.1−48.63        | 6.5−109.5         | 1.0           |
| Mean STB                                       |      | 19.208            | 20.643            |               |         |
| SD                                             |      | 8.926             | 18.127            |               |         |
| Conjugated bilirubin (CB)                      | umol/L| 2.4−16.4          | 2.3−99.2          | 1.0           |
| Mean CB                                        |      | 6.923             | 9.408             |               |         |
| SD                                             |      | 3.542             | 17.208            |               |         |
| Unconjugated bilirubin (UCB)                   | umol/L| 6−34.13           | 3.3−29.3          | 1.0           |
| Mean UCB                                       |      | 12.284            | 11.182            |               |         |
| SD                                             |      | 5.951             | 4.921             |               |         |
| HBV-DNA                                        | IU/mL| 5.12E+03−1.12E+08 | 6.34E+04−9.40E+08 | 0.0096        |
| Mean HBV-DNA                                   |      | 2.521E+07         | 5.655E+07         |               |         |
| SD                                             |      | 3.221E+07         | 1.717E+08         |               |         |

3. Results

3.1. Diagnosis of the Patients. To study serum protein biomarkers in LSSD and DHSM CHB patients, we obtained a total of 80 participants, including 30 LSSD-CHB, 30 DHSM-CHB patients, and 20 healthy volunteers. As shown in Table I and Table S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/3290260, mean ages of LSSD-CHB, DHSM-CHB, and HCTL were 30, 36.8, and 35.5 years, respectively. Except missing information of three, all patients were positive to HBsAg and anti-HBc. There were two LSSD-CHB patients positive to anti-HBs, and 9 LSSD-CHB and 12 DHSM-CHB patients positive to anti-HBe. HBV-DNA levels in the blood samples of LSSD-CHB and DHSM-CHB patients were ranged from 5.12E+03−1.12E+08 IU/mL and 6.34E+04−9.40E+08 IU/mL, respectively. It is interesting that hepatitis B viral load (HBV-DNA copies) was significantly different (p = 0.0096) in LSSD-CHB and DHSM-CHB patients. Next, we examined ALT, AST, STB, CB, and UCB levels in the blood samples of CHB patients. Mean values of
these diagnosis biomarkers in the blood samples of LSSD-CHB and DHSM-CHB patients were similar. In addition, the levels of ALT and AST remained at a high level, compared to healthy individuals [26, 27], which confirmed their CHB infection.

3.2. Protein Identification and Quantification by iTRAQ. Next, we quantified the serum proteins in these LSSD-CHB and DHSM-CHB patients using iTRAQ. Initially, a total of 371,034 spectra were generated by liquid chromatography coupled to mass spectrometry (LC-MS/MS) analysis. Of them, 98,243 spectra (5,591 unique peptides) were aligned to 842 proteins from 666 families. The mass distribution of identified proteins (Figure 1(a)) suggested by Mascot revealed 170 (98.69%) were above 10 kDa, of which 170 (20.19%) and 141 (16.75%) were 10 to 20 kDa and above 100 kDa, respectively. We also counted the proteins aligned with significant peptides, shown in Figure 1(b), and 547 (64.96%) proteins were aligned by two and more peptides. In addition, the distribution of protein sequence coverage is shown in Figure 1(c). Protein sequence coverage with 40–100%, 30–40%, 20–30%, 10–20%, and under 10% variation accounted for 8.79%, 14.25%, 17.70%, 23.28%, and 35.99%, respectively. In Figure 1(d), we showed correlation between two samples and found LSSD-CHB samples were closer to DHSM-CHB samples than HCTL.

3.3. Identification of Differentially Expressed Proteins. Differentially expressed proteins were defined as those showed greater than 1.2-fold change in relative abundance and a p value < 0.05. Compared to HCTL we identified a total of 392 proteins differentially expressed (Table S2), of which 273 were identified in LSSD-CHB group and 345 in DHSM-CHB group. As shown in the volcano plots, we identified 172
upregulated and 101 downregulated proteins in LSSD-CHB group (Figure 2(a)) and 199 upregulated and 146 downregulated proteins in DHSM-CHB group (Figure 2(b)), compared to HCTL group. Venn diagram (Figure 2(c)) revealed LSSD-CHB and DHSM-CHB shared 142 upregulated and 84 downregulated proteins; 30 and 57 proteins were exclusively upregulated in LSSD-CHB and DHSM-CHB, respectively; 17 and 62 proteins were exclusively downregulated in LSSD-CHB and DHSM-CHB, respectively; and no protein was identified with upregulation in one CHB subtype but with downregulation in another.

3.4. Potential Biomarkers for CHB. The identification of proteins differentially expressed in LSSD-CHB and DHSM-CHB groups relative to the HCTL group was of interest as these could provide leads for potentially useful diagnostic and prognostic biomarkers. First, we examined those 142 commonly upregulated and 84 commonly downregulated proteins. As shown in Table 2, the largest upregulated protein family was immunoglobulin related protein, showing 20 upregulated and 3 downregulated proteins identified. In clinical immunology, levels of immunoglobulins especially IgG can be used to characterize viral hepatitis in patients [28, 29]. Four IgG subclasses (IgG1 to IgG4) differ in their heavy chain constant regions and have different effects on virus-cell fusion inhibition, virus neutralization, and overall course of infection, as have been reported for various viruses including HIV [30] and HBV [31]. Highly expressed proteins encoding heavy chains for immunoglobulins including IGHG1, IGHG3, IGHG4, and IGH@ have been reported.
| Family                  | UniProt ID   | Gene name       | Description                                      | Number of significant matches | Number of significant sequences | emPAI | FC<sup>a</sup> | p value<sup>b</sup> | FC<sup>a</sup> | p value<sup>b</sup> |
|------------------------|--------------|-----------------|--------------------------------------------------|------------------------------|-------------------------------|-------|----------------|-----------------|----------------|-----------------|
| Immunoglobulin related proteins | A0A087WV47_HUMAN | IGHG1 | Ig gamma-1 chain C region | 1541 | 14 | 5.37 | 1.84 | 6.30E−05 | 1.47 | 2.71E−04 |
|                        | A0A087WV45_HUMAN | IGHG1 | Ig gamma-1 chain C region | 1501 | 14 | 4.82 | 1.87 | 3.51E−05 | 1.48 | 1.69E−04 |
|                        | A0A087XIC7_HUMAN | IGHG1 | Ig gamma-1 chain C region | 1501 | 13 | 4.79 | 1.84 | 6.12E−05 | 1.47 | 2.60E−04 |
|                        | A0A087WXL8_HUMAN | IGHG3 | Ig gamma-3 chain C region | 739 | 11 | 2.07 | 1.84 | 5.45E−05 | 1.47 | 1.12E−04 |
|                        | A0A0G2JD4_HUMAN | IGHG4 | Ig gamma-4 chain C region (fragment) | 31 | 6 | 1.56 | 1.69 | 6.47E−04 | 1.24 | 4.12E−02 |
|                        | KV112_HUMAN   | | Ig kappa chain V-I region Kue | 2 | 2 | 0.53 | 1.82 | 1.39E−03 | 1.51 | 9.53E−04 |
|                        | KV31_HUMAN    | | Ig kappa chain V-III region IARC/BL4I | 2 | 1 | 0.49 | 1.61 | 5.86E−03 | 1.28 | 1.64E−02 |
|                        | LY302_HUMAN   | | Ig lambda chain V-III region LOI | 25 | 3 | 4.01 | 1.73 | 1.75E−04 | 1.33 | 8.79E−04 |
|                        | LY303_HUMAN   | | Ig lambda chain V-III region SH | 6 | 1 | 0.61 | 1.99 | 1.95E−07 | 1.56 | 5.32E−07 |
|                        | S6B26_HUMAN   | | IgG H chain | 384 | 3 | 0.6 | 2.05 | 1.06E−04 | 1.57 | 9.46E−04 |
|                        | S6BAG6_HUMAN  | | IgG H chain | 389 | 4 | 0.9 | 2.05 | 4.60E−05 | 1.56 | 5.55E−04 |
|                        | S6BGE0_HUMAN  | | IgG H chain | 394 | 4 | 1.13 | 2.04 | 4.84E−05 | 1.56 | 6.39E−04 |
|                        | S6B14_HUMAN   | | IgG H chain | 384 | 3 | 0.73 | 2.05 | 9.63E−05 | 1.57 | 5.91E−04 |
|                        | S6C4R_HUMAN   | | IgL chain | 257 | 6 | 6.68 | 1.53 | 2.61E−02 | 1.27 | 4.54E−02 |
|                        | Q6GMY6_HUMAN  | | IGH@ protein | 1524 | 14 | 6.04 | 1.83 | 6.27E−05 | 1.46 | 3.15E−04 |
|                        | A0A0F77Q8_HUMAN | IGHV4-4 | IGHV4-4 protein (fragment) | 3 | 2 | 1.27 | 1.49 | 9.67E−03 | 1.24 | 2.27E−02 |
|                        | Q5FW9_HUMAN   | | IGL@ protein | 143 | 6 | 6.03 | 1.60 | 1.35E−02 | 1.32 | 2.83E−02 |
|                        | Q6PIK1_HUMAN  | | IGL@ protein | 197 | 5 | 4.45 | 1.59 | 1.37E−02 | 1.29 | 4.62E−02 |
|                        | Q9UL84_HUMAN  | | Myosin-reactive immunoglobulin heavy chain variable region (fragment) | 6 | 3 | 1.69 | 1.52 | 1.58E−02 | 1.46 | 2.04E−04 |
|                        | Q9UL79_HUMAN  | | Myosin-reactive immunoglobulin light chain variable region (fragment) | 2 | 1 | 0.57 | 0.65 | 6.56E−04 | 0.50 | 1.93E−12 |
|                        | A0A0CDH33_HUMAN | IGHV1-24 | Protein IGHV1-24 (fragment) | 5 | 2 | 0.84 | 0.82 | 5.43E−03 | 0.81 | 1.38E−02 |
|                        | A0A0AM714_HUMAN | IGV1-16 | Protein IGKVI-16 (fragment) | 5 | 1 | 0.9 | 1.58 | 3.55E−03 | 1.26 | 3.22E−02 |
|                        | A0A07S639_HUMAN | IGLV7-46 | Protein IGLV7-46 (fragment) | 31 | 3 | 1.41 | 1.54 | 9.85E−03 | 1.36 | 2.46E−03 |
| Family          | UniProt_ID | Gene_name         | Description                                      | Number of significant matches | Number of significant sequences | emPAI | SSD-CHB versus HCTL FCa | SSDHS-CHB versus HCTL FCa | p valueb |
|-----------------|------------|-------------------|--------------------------------------------------|------------------------------|-------------------------------|-------|-------------------------|---------------------------|---------|
| Complement      | B7Z1F8_HUMAN | B7Z1F8_HUMAN      | cDNA FLJ33023, highly similar to complement C4-B | 2330                         | 12                            | 56.68 | 0.78                   | 1.27 x 10^-3            | 0.77    | 2.33 x 10^-3          |
|                 | B4E356_HUMAN | B4E356_HUMAN      | cDNA FLJ58413, highly similar to complement component C7 | 63                           | 10                            | 1.74  | 0.55                   | 8.69 x 10^-10           | 0.50    | 8.03 x 10^-15         |
|                 | A8K2T4_HUMAN | A8K2T4_HUMAN      | cDNA FLJ78207, highly similar to human complement protein component C7 mRNA | 86                           | 15                            | 1.31  | 0.55                   | 1.62 x 10^-9            | 0.50    | 2.39 x 10^-13         |
|                 | B2RA39_HUMAN | B2RA39_HUMAN      | cDNA, FLJ94686, highly similar to Homo sapiens complement factor H-related 5 (CFHLS), mRNA | 35                           | 5                             | 0.51  | 0.54                   | 7.03 x 10^-12           | 0.57    | 1.44 x 10^-11         |
|                 | CO4A_HUMAN  | CO4A              | Complement C4-A                                  | 5889                         | 65                            | 21.28 | 0.79                   | 2.87 x 10^-3            | 0.75    | 7.70 x 10^-4          |
|                 | CO4B_HUMAN  | CO4B              | Complement C4-B                                  | 5983                         | 66                            | 23.03 | 0.79                   | 2.95 x 10^-3            | 0.74    | 5.91 x 10^-4          |
|                 | A0A024R035_HUMAN | A0A024R035_HUMAN  | Complement component 9, isoform CRA.a         | 225                          | 17                            | 5.12  | 0.67                   | 1.36 x 10^-5            | 0.54    | 6.61 x 10^-13         |
|                 | CO6_HUMAN   | CO6               | Complement component C6                         | 92                           | 12                            | 0.83  | 0.50                   | 3.34 x 10^-12           | 0.48    | 4.06 x 10^-15         |
|                 | CO8A_HUMAN  | CO8A              | Complement component C8 alpha chain             | 66                           | 8                             | 1.51  | 0.66                   | 1.94 x 10^-6            | 0.53    | 8.36 x 10^-15         |
|                 | F5GY80_HUMAN | F5GY80_HUMAN      | Complement component C8 beta chain             | 135                          | 16                            | 3.28  | 0.65                   | 6.59 x 10^-7            | 0.61    | 1.07 x 10^-9          |
|                 | CO8G_HUMAN  | CO8G              | Complement component C8 gamma chain           | 41                           | 8                             | 6.86  | 0.61                   | 8.80 x 10^-8            | 0.50    | 3.38 x 10^-14         |
| Family | UniProt_ID | Gene name | Description | Number of significant matches | Number of significant sequences | emPAI | LSSD-CHB versus HCTL | SSDHS-CHB versus HCTL |
|--------|------------|-----------|-------------|-----------------------------|--------------------------------|-------|---------------------|----------------------|
|        | B1AKG0_HUMAN | CFHR1     | Complement factor H-related protein 1 | 216                          | 6                              | 4.26  | 0.66                | 1.30E-06             | 0.75                | 4.80E-04             |
|        | FHR3_HUMAN | CFHR3     | Complement factor H-related protein 3 | 45                           | 6                              | 1.19  | 0.82                | 7.05E-03             | 0.77                | 2.36E-03             |
|        | FHR4_HUMAN | CFHR4     | Complement factor H-related protein 4 | 31                           | 3                              | 0.41  | 0.37                | 3.90E-23             | 0.31                | 1.62E-44             |
|        | A0A052445_HUMAN | CFP      | Complement factor properdin isoform 1 (fragment) | 13                           | 2                              | 0.17  | 0.58                | 2.21E-11             | 0.72                | 2.90E-05             |
| Apolipoprotein |        | APOA2_HUMAN | Apolipoprotein A-II | 42                           | 2                              | 1.84  | 1.48                | 4.64E-02             | 1.26                | 3.72E-02             |
|        | A0A054507_HUMAN | APOA5    | Apolipoprotein A-V, isoform CRA_a | 15                           | 7                              | 1     | 0.73                | 2.03E-05             | 0.62                | 2.95E-08             |
|        | EIB59_HUMAN | APOB     | Apolipoprotein B (fragment) | 129                          | 7                              | 10.94 | 1.97                | 1.23E-05             | 1.53                | 9.70E-05             |
|        | C0J2Y2_HUMAN | APOB     | Apolipoprotein B (including AgX antigen) | 5227                         | 215                            | 21.89 | 1.84                | 2.08E-04             | 1.44                | 1.07E-03             |
|        | Q59HB3_HUMAN | APOC3    | Apolipoprotein B variant (fragment) | 1661                         | 69                             | 16.33 | 1.85                | 2.79E-04             | 1.41                | 1.40E-03             |
|        | B0YI2_HUMAN | APOC4    | Apolipoprotein C-III | 577                          | 5                              | 8.48  | 0.66                | 1.44E-05             | 0.55                | 3.02E-09             |
|        | A0A0471_HUMAN | APOC4    | Apolipoprotein C-IV | 26                           | 5                              | 3.84  | 0.80                | 1.35E-03             | 0.60                | 8.22E-09             |
| Histone |        | H1S_HUMAN | HIST1H1B | Histone H1.5 | 4                              | 2                              | 0.35  | 2.02                | 8.32E-06             | 1.72                | 1.02E-04             |
|        | A0A040107_HUMAN | HIST1H2AC | Histone H2A | 21                           | 4                              | 2.27  | 4.67                | 1.78E-28             | 3.63                | 4.54E-33             |
|        | C0J2Y2_HUMAN | H2AFV    | Histone H2A | 13                           | 3                              | 1.1   | 4.84                | 3.09E-29             | 3.73                | 2.99E-37             |
|        | A0A024527_HUMAN | HIST1H2BD | Histone H2B | 12                           | 4                              | 1.15  | 4.58                | 1.39E-29             | 3.38                | 6.00E-31             |
|        | B2R4P9_HUMAN | HIF3A    | Histone H3 | 9                            | 3                              | 0.88  | 4.98                | 5.44E-32             | 3.72                | 9.13E-39             |
|        | Q597EC_HUMAN | HIST2H3PS2 | Histone H3 | 4                            | 3                              | 0.6   | 2.59                | 1.10E-08             | 1.87                | 3.39E-06             |
|        | B2R4R0_HUMAN | HIST1H4 | Histone H4 | 39                           | 5                              | 13.27 | 4.47                | 3.83E-35             | 3.41                | 3.84E-45             |
| Heat shock protein |        | A0A024480_HUMAN | HSP90AB1 | Heat shock protein 90 kDa alpha (cytosolic), class B member 1, isoform CRA_a | 12                          | 6                              | 0.31  | 1.46                | 3.59E-02             | 1.58                | 2.77E-05             |
|        | HS90A_HUMAN | HSP90AA1 | Heat shock protein HSP 90-alpha | 28                           | 10                             | 0.55  | 1.58                | 5.45E-03             | 1.65                | 3.81E-07             |
| Family                        | UniProtID         | Gene name         | Description                                                                 | Number of significant matches | Number of significant sequences | emPAI | LSSD-CHB versus HCTL PC | SSDHS-CHB versus HCTL PC |
|-------------------------------|-------------------|-------------------|------------------------------------------------------------------------------|-------------------------------|--------------------------------|-------|------------------------|------------------------|
| Insulin-like growth factor binding protein | CIK3N, HUMAN      | IGFBP1            | Insulin-like growth factor binding protein 1 (fragment)                      | 3                             | 2                              | 0.22  | 0.61                   | 2.62'E-07              | 0.60                   | 5.40'E-09              |
|                               | A0A024RIU8, HUMAN  | IGFBP4            | Insulin-like growth factor binding protein 4, isoform CRA_a                  | 2                             | 1                              | 0.1   | 0.66                   | 8.52'E-06              | 0.66                   | 1.00'E-06              |
|                               | A0A024R433, HUMAN  | IGFBP5            | Insulin-like growth factor binding protein 5, isoform CRA_a                  | 3                             | 2                              | 0.18  | 0.83                   | 3.37'E-03              | 0.80                   | 4.98'E-02              |
| Ras-related protein           | A0A024RI713, HUMAN | RAB8A             | RAB8A, member RAS oncogene family, isoform CRA_a                             | 11                            | 4                              | 1.08  | 1.75                   | 1.87'E-03              | 1.73                   | 9.62'E-06              |
|                               | A0A024RB87, HUMAN  | RAP1B             | RAP1B, member of RAS oncogene family, isoform CRA_a                           | 10                            | 4                              | 1.66  | 1.85                   | 8.55'E-05              | 1.96                   | 7.63'E-11              |
|                               | RAB10, HUMAN       | RAB10             | Ras-related protein Rab-10                                                  | 9                             | 3                              | 0.71  | 1.74                   | 2.50'E-03              | 1.72                   | 2.60'E-05              |
|                               | RAB1B, HUMAN       | RAB1B             | Ras-related protein Rab-1B                                                  | 5                             | 4                              | 0.73  | 1.91                   | 3.03'E-05              | 1.57                   | 1.03'E-05              |
|                               | RAB1B, HUMAN       | RAB1B             | Ras-related protein Rab-1B                                                   | 5                             | 4                              | 0.73  | 1.91                   | 3.03'E-05              | 1.57                   | 1.03'E-05              |
|                               | RAB27B, HUMAN      | RAB27B            | Ras-related protein Rab-27B                                                  | 5                             | 4                              | 0.73  | 1.91                   | 3.03'E-05              | 1.57                   | 1.03'E-05              |
|                               | RAB7A, HUMAN       | RAB7A             | Ras-related protein Rab-7A                                                   | 16                            | 6                              | 1.94  | 1.60                   | 2.16'E-02              | 1.66                   | 1.48'E-04              |
| Serum amyloid                 | D3DQX7, HUMAN      | SAA1              | Serum amyloid A protein                                                       | 59                            | 5                              | 9.88  | 0.33                   | 2.58'E-13              | 0.40                   | 3.36'E-24              |
|                               | SAAL HUMAN         | SAA1              | Serum amyloid A-1 protein                                                     | 40                            | 4                              | 4.99  | 0.48                   | 1.21'E-15              | 0.38                   | 4.55'E-28              |
|                               | SAAL HUMAN         | SAA2              | Serum amyloid A-2 protein                                                     | 24                            | 4                              | 4.99  | 0.48                   | 7.27'E-16              | 0.36                   | 3.08'E-31              |
|                               | SAMP, HUMAN        | APGS              | Serum amyloid P-component                                                    | 228                           | 8                              | 15.23 | 0.83                   | 8.13'E-03              | 0.70                   | 4.45'E-05              |
| von Willebrand factor         | LE8E35, HUMAN      | VWF               | von Willebrand factor                                                        | 189                           | 38                             | 0.98  | 1.68                   | 2.69'E-03              | 1.41                   | 9.67'E-04              |
|                               | VWF, HUMAN         | VWF               | von Willebrand factor                                                        | 186                           | 38                             | 0.93  | 1.67                   | 3.05'E-03              | 1.41                   | 7.94'E-04              |

*a* Fold change provided by MASCOT.

*b* P values calculated by edgeR to show the significance of different expression.
with upregulation in HBV [32, 33] and HCC patients [34]. Other upregulated protein families such as heat shock protein, histone, ras-related protein, and von Willebrand factor identified in current study have also been reported in patients infected by HBV or hepatitis C virus (HCV) [35–38]. The largest downregulated protein family was complement, 15 complement proteins downregulated in LSSD-CHB with 0.82- to 0.37-fold change and in DHSM-CHB with 0.77- to 0.31-fold change. Other protein families like insulin-like growth factor binding protein and serum amyloid protein were also decreased in CHB patients in comparison to HCTL group. In addition, several known upregulated proteins from other families in patients infected by HBV or HCV (Table 2), such as apolipoproteins (APOA2, APOB, and APOB-variant) [39, 40], A2M (alpha-2-macroglobulin) [41], alpha-actinin-3 (ACTN3) [42, 43], vimentin (VIM) [38], and putative uncharacterized proteins (DFKZp686N02209 and DFKZp686I04196) [34, 44, 45], were identified in LSSD-CHB and DHSM-CHB groups. The different expression of proteins in the serum of CHB patients indicates they may have functions in response of HBV and CHB processing and can be used as biomarkers in clinical diagnosis.

We next analyzed the potential functions of commonly differentially expressed serum proteins in LSSD-CHB and DHSM-CHB groups using DAVID Bioinformatics Resources 6.7 [24] and STRING v10 [25]. Cellular component annotation (Figure 3(a)) showed 63 and 7 proteins were “extracellular region” (GO: 0005576, GO: 0005615, and GO: 0044421) and “lipids” (GO: 0032994 and GO: 0034358), respectively. However biological process annotation (Figure 3(b)) showed most of the differentially expressed proteins associated with immune response, including “acute inflammatory response” (GO: 0002526), “response to wounding” (GO: 0009611), “inflammatory response” (GO: 0006954), “complement activation” (GO: 0006956), “defense response” (GO: 0006959), “B cell mediated immunity” (GO: 0019724), and “protein processing” (GO: 0002252).

It has been well studied that immunological events are necessary to control hepatitis B virus (HBV) infection [46, 47]. In addition, KEGG pathway analysis also showed differentially expression proteins function mainly in the pathways of “complement and coagulation cascades” (hsa04610), “systemic lupus erythematosus” (hsa05322), “focal adhesion” (hsa04510), and “viral carcinogenesis” (hsa05203). Overall, differentially expressed proteins in both LSSD-CHB and DHSM-CHB groups have potential ability to be used as biomarkers.

3.5. Dysregulated Proteins Detected Exclusively in LSSD-CHB and DHSM-CHB. Next, we examined differentially
expressed proteins exclusively in LSSD-CHB and DHSM-CHB groups. A total of 30 upregulated and 17 downregulated proteins were specifically identified in LSSD-CHB patient serum samples (Table 3). Among them 11 upregulated immunoglobulin related proteins, gelsolin (GSN), serum paraoxonase/lactonase 3 (PON3), likely SNC73 protein, transient receptor potential cation channel subfamily M member 8 (TRPM8), and several uncharacterized proteins (DKFZp686M08189, DKFZp686C02220, and DKFZp686K04218) attracted our attention due to their high abundance. Serum PON3 concentrations have been reported to increase in patients with CHB or cirrhosis and showed significant direct correlations with the degree of periportal abnormalities including fibrosis and with serum FAS (a marker of antiapoptosis) concentrations [48]; however, serum gelsolin level has been reported to reduce significantly in patients with acute liver failure (47%), myocardial infarction (69%), sepsis (51%), and myonecrosis (66%) [49]. Among the specifically downregulated serum proteins in LSSD-CHB patients fibulin-1 (FBLN1) is a tumor suppressor in hepatocellular carcinoma [50]. Proteins specifically differentially expressed in LSSD-CHB patients were predicted to function mainly in biological processes of "protein activation cascade" (GO: 00072376), "regulation of response to wounding" (GO: 1903034), "blood coagulation, fibrin clot formation" (GO: 0027378), "negative regulation of response to stimulus" (GO: 0048585), and "acute-phase response" (GO: 0006953).

We also identified 57 upregulated and 62 downregulated proteins exclusively in DHSM-CHB patients (Table 4). Two IGL@proteins (Q6GMX4_HUMAN and Q6PIQ7_HUMAN) were specifically upregulated in DHSM-CHB patients with 1.27-fold change. Transthyretin (TTR), upregulated 1.34-fold in DHSM-CHB, can be induced by hepatitis C virus and activate TGF-β signaling pathway with furin [51]. Interestingly, we found three members of tubulin (TUBA4A, TUBB1, and TUBB8) were upregulated only in DHSM-CHB patients compared with HCTL. Although there are few reports about tubulin and HBV, it is well known that 42 kDa tubulin alpha-6 chain fragment in well-differentiated hepatocellular carcinoma tissues is from patients infected with HCV [52]. In addition, we found actinin, alpha 1 (ACTN1), which can directly interact with HCV [53], GAPDH, which can bind to the HBV posttranscriptional regulatory element [54], and polymeric immunoglobulin receptor (PIGR), the main transporter of IgA [55], were upregulated in DHSM-CHB but not in LSSD-CHB. Among DHSM-CHB specifically downregulated proteins we identified three members of keratin type I (KRT9, KRT10, and KRT14) and another three members of keratin type II (KRT1, KRT2, and KRT6B). Although there is no evidence showing relation between these six keratin proteins with CHB or other liver diseases, variant keratins are associated with progression of fibrosis during chronic hepatitis C infection [56]. Differentially expressed proteins exclusively detected in DHSM-CHB patients were predicted to be involved in the biological processes of "immune system process" (GO: 0002376), "response to stress" (GO: 0006950), "defense response" (GO: 0006952), "immune response" (GO: 0006955), and "single-organism metabolic process" (GO: 0044710).

Compared to HCTL group up- and downregulated proteins exclusively in LSSD-CHB and DHSM-CHB patients showed their potential ability of being biomarkers for these two subtypes of HBV induced CHB. Some of them have been reported in other studies; however, more experiments need to be performed to investigate their functions and validate their specificity and accuracy in clinical trials.

3.6. Validation of the Quantitative Proteomic Analysis. To validate the results obtained by proteomics analysis, eight randomly selected proteins and internal control albumin with altered expression profile were monitored by western blotting in an independent group of samples. Figures 4(a) and 4(b) showed the western blots for eight proteins and internal control albumin. PSMA6 (20S proteasome alpha6), PSMA7 (20S proteasome alpha7/alpha8) were upregulated and PF4 V (platelet factor 4 variant) was downregulated in LSSD-CHB group compared to HCTL (Figure 4(c)). Except SERPING1 (plasma protease CI inhibitor), AHSG (fetuin-A), ACTB (actin), CTSC (cathepsin C), and PLTP (phospholipid transfer protein) were upregulated in the serum of DHSM-CHB patients (Figure 4(d)). Although the difference between patients and healthy participants was not significant by western blotting analysis, their regulations in patients and healthy group were consistent with iTRAQ. The original images of western blots (see Figure S1) might contain some differences due to brightness and contrast settings.

4. Discussion

Quantitation of serum or plasma proteins using comparative proteomics has recently been suggested as a suitable approach for the detection of liver disease biomarkers [17, 57–59]. The iTRAQ technology has been proposed as a powerful alternative to common tools (e.g., ELISA) and a flurry of applications emerged in the literature.

In this study, iTRAQ LC–MS/MS proteomics was used to detect serum protein as biomarkers of LSSD-CHB and DHSM-CHB patients. We compared the proteomics profile of LSSD-CHB and DHSM-CHB patients with healthy individuals and indicated 142 upregulated and 84 downregulated proteins shared by these two CHB subtype diseases. Protein-protein interaction network (Figure 5) showed several significant proteins might function in response to HBV, such as actins (ACTA2, ACTB, ACTBL2, ACTN3, and ACTN4), apolipoproteins (APOA2, APOA5, APOB, APOC3, and APOC4), heat shock proteins (HSP90AA1 and HSP90AB1), and proteasome subunit proteins (PSMA1 and PSMA4). It has been reported that HBV core proteins can interact with the C-terminal region of actin-binding protein [60] and HBV X protein (HBx) can block filamentous actin bundles by interaction with eEF1A1 (eukaryotic translation elongation factor 1 alpha 1) [61]. In addition, ACTA2 is a marker of hepatitis stellate cells and correlated significantly with necroinflammatory grades and fibrotic stages in CHB or CHC [13]. Apolipoproteins are supposed to enhance the infectivity of hepatitis virus during the infection [39, 62] and
Table 3: Up- and downregulated serum proteins specifically in LSSD-CHB.

| UniProtID       | Gene name | Description                                                                 | Number of significant matches | Number of significant sequences | emPAI | FC^a | p value^b |
|-----------------|-----------|-----------------------------------------------------------------------------|-------------------------------|--------------------------------|-------|------|-----------|
| **Upregulated** |           |                                                                             |                               |                                |       |      |           |
| A0A024R6I9_HUMAN| SERPINA4  | Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 4, isoform CRA_a | 79                             | 12                              | 3.79  | 1.78 | 5.24E - 05|
| A0A075B6K3_HUMAN| IGLV2-11  | Protein IGLV2-II (fragment)                                                  | 8                             | 1                               | 0.9   | 1.43 | 4.94E - 02|
| A0A075B6K6_HUMAN| IGLV4-3   | Protein IGLV4-3                                                             | 4                             | 1                               | 0.51  | 1.54 | 1.23E - 02|
| A0A075B6N7_HUMAN| IGHA2     | Ig alpha-2 chain C region (fragment)                                         | 80                            | 6                               | 1.94  | 1.44 | 4.71E - 02|
| A0A0A0MS5L_HUMAN| GSN       | Gelsolin                                                                    | 146                           | 16                              | 2.42  | 1.51 | 2.86E - 02|
| A0A0B4JHV4_HUMAN| IGHV1-46  | Protein IGHV1-46 (fragment)                                                  | 2                             | 1                               | 0.23  | 1.44 | 4.22E - 02|
| A0N7J6_HUMAN    | REV25-2   | REV25-2 (fragment)                                                           | 2                             | 2                               | 0.47  | 1.92 | 3.69E - 03|
| A0N8J1_HUMAN    | NG9 gene from fetal liver DNA (fragment) Carboxypeptidase N subunit 2        | 6                             | 1                               | 1.1                             | 1.49  | 1.49 | 1.49E - 02|
| CPN2_HUMAN      | CPN2      | Kininogen 1, isoform CRA_a                                                   | 94                            | 11                              | 2.09  | 1.48 | 1.88E - 02|
| D3DNU8_HUMAN    | KNG1      | KNG1                                                                         | 91                            | 14                              | 3.3   | 1.50 | 1.14E - 02|
| FETUA_HUMAN     | AHSG      | Alpha-2-HS-glycoprotein                                                      | 57                            | 3                               | 0.64  | 1.70 | 1.36E - 03|
| FETUB_HUMAN     | FETUB     | Fetuin-B                                                                    | 9                             | 3                               | 0.29  | 1.65 | 6.32E - 04|
| HV208_HUMAN     | V(k)3       | Ig alpha-2 chain C region SESS                                               | 2                             | 1                               | 0.21  | 2.27 | 1.25E - 08|
| ITB1_HUMAN      | ITGB1     | Integrin beta-1                                                             | 3                             | 2                               | 0.06  | 1.46 | 3.43E - 02|
| KV19_HUMAN      | Ig kappa chain V-I region Wes                                               | 21                            | 2                               | 2.16  | 1.52 | 6.37E - 03|
| KV308_HUMAN     | Ig kappa chain V-III region CLL                                              | 4                             | 1                               | 0.49  | 1.65 | 8.16E - 03|
| LV204_HUMAN     | Ig lambda chain V-II region TRO                                              | 7                             | 2                               | 0.96  | 1.59 | 1.25E - 02|
| UniProtID       | Gene name          | Description                                                                 | Number of significant matches | Number of significant sequences | emPAI | FC   | \(p\) value |
|-----------------|--------------------|------------------------------------------------------------------------------|------------------------------|--------------------------------|-------|------|-------------|
| PCYOX_HUMAN     | PCYOX1             | Prenylcysteine oxidase 1 / Pigment                                            | 48                           | 9                              | 1.89  | 1.43 | 3.70E – 02 |
| PEDF_HUMAN      | SERPINFI           | Pigment epithelium-derived factor                                             | 23                           | 9                              | 1.54  | 1.48 | 1.56E – 02 |
| PON3_HUMAN      | PON3               | Serum paraoxonase/lactonase 3                                                | 179                          | 12                             | 3.89  | 1.51 | 4.11E – 02 |
| Q6MZX9_HUMAN    | DKFZp686M08189     | Putative uncharacterized protein                                              | 124                          | 7                              | 1.41  | 1.43 | 3.95E – 02 |
| Q6N09L_HUMAN    | DKFZp686C02220     | Putative uncharacterized protein                                              | 71                           | 6                              | 1.2   | 1.46 | 2.31E – 02 |
| Q6ZVX0_HUMAN    | DKFZp686M08189     | Putative uncharacterized protein                                              | 125                          | 7                              | 1.38  | 1.43 | 4.13E – 02 |
| Q7Z379_HUMAN    | DKFZp686K04218     | Putative uncharacterized protein                                              | 124                          | 7                              | 1.44  | 1.43 | 3.96E – 02 |
| Q8TE63_HUMAN    |                    | Immunoglobulin light chain variable region (fragment)                         | 4                            | 2                              | 0.97  | 1.45 | 4.51E – 02 |
| Q96K68_HUMAN    |                    | Highly similar to *Homo sapiens* SNC73 protein (SNC73) mRNA                  | 205                          | 9                              | 1.77  | 1.45 | 2.93E – 02 |
Table 3: Continued.

| UniProtID      | Gene_name      | Description                                                                 | Number of significant matches | Number of significant sequences | emPAI | FC | p value  |
|----------------|----------------|------------------------------------------------------------------------------|-------------------------------|--------------------------------|-------|----|----------|
| Q9NPP6_HUMAN   |                | Immunoglobulin heavy chain variant (fragment)                                | 125                           | 7                              | 1.75  | 1.43| 4.92E-02 |
| Q9UL83_HUMAN   |                | Myosin-reactive immunoglobulin light chain variable region (fragment)        | 14                            | 2                              | 2.98  | 1.54| 5.43E-03 |
| Q9UL89_HUMAN   |                | Myosin-reactive immunoglobulin heavy chain variable region (fragment)        | 18                            | 5                              | 8.63  | 1.49| 2.16E-02 |
| TRPM8_HUMAN    | TRPM8          | Transient receptor potential cation channel subfamily M member 8             | 3                             | 2                              | 0.04  | 1.60| 6.39E-03 |
| A0A024QZK7_HUMAN | HK1            | Hexokinase                                                                  | 5                             | 1.00E + 00                     | 0.03  | 0.82| 7.31E-03 |
| A0A024R2X3_HUMAN | HYAL1          | Hyaluronidase                                                               | 12                            | 5.00E + 00                     | 0.55  | 0.73| 2.82E-05 |
| A0A024R45L_HUMAN | SERPINE2       | Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2, isoform CRA_a Complement component 1, q subcomponent, A chain, isoform CRA_a Lectin galactoside-binding soluble 3 binding protein isoform 1 (fragment) | 17                            | 8                              | 1.05  | 0.83| 2.36E-03 |
| A0A024RAG6_HUMAN | CIQA           | Complement component 1, q subcomponent, A chain, isoform CRA_a Lectin galactoside-binding soluble 3 binding protein isoform 1 (fragment) | 12                            | 4                              | 1.08  | 0.75| 4.11E-04 |
| A0A0S2Z3YL_HUMAN | LGALS3BP       | Galactoside-binding soluble 3 binding protein isoform 1 (fragment)          | 247                           | 14                             | 4.17  | 0.71| 2.49E-06 |
| A0A0S2Z4D4_HUMAN | PLPI           | Proteolipid protein 1 isoform 1 (fragment)                                  | 2                             | 1                              | 0.11  | 0.40| 1.65E-04 |
| UniProt_ID   | Gene_name  | Description                                      | Number of significant matches | Number of significant sequences | emPAI | FC<sup>a</sup> | p value<sup>b</sup> |
|-------------|------------|--------------------------------------------------|-------------------------------|--------------------------------|-------|-------------|------------------|
| ADT4_HUMAN  | SLC25A31   | ADP/ATP translocase 4                            | 5                             | 1                              | 0.16  | 0.65        | 1.28E - 02      |
| B1AHL2_HUMAN| FBLN1      | Fibulin-1                                        | 74                            | 8                              | 0.92  | 0.78        | 4.33E - 04      |
| CALU_HUMAN  | CALU       | Calumenin                                        | 12                            | 7                              | 0.86  | 0.76        | 2.45E - 04      |
| CFAH_HUMAN  | CFH        | Complement factor H Lipopolysaccharide-binding   | 689                           | 31                             | 4.02  | 0.83        | 8.87E - 03      |
| LBP_HUMAN   | LBP        | Lipopolysaccharide-binding protein               | 148                           | 11                             | 3.18  | 0.79        | 3.74E - 04      |
| LRPL_HUMAN  | LRP1       | Prolow-density lipoprotein receptor-related      | 37                            | 20                             | 0.16  | 0.83        | 7.44E - 03      |
| LTBPL_HUMAN | LTBPI      | Latent-transforming growth factor beta-binding   | 19                            | 8                              | 0.16  | 0.77        | 8.36E - 04      |
| NUCB1_HUMAN | NUCB1      | Nucleobindin-1                                   | 17                            | 7                              | 0.66  | 0.78        | 5.80E - 04      |
| Q8NBH6_HUMAN| Fibulin-1  |                                              | 125                           | 11                             | 2.25  | 0.75        | 1.26E - 04      |
| Q9HCC1_HUMAN| Single chain Fv (fragment)                      | 10                            | 2                              | 0.96  | 0.79        | 4.29E - 04      |
| THR_B_HUMAN | F2         | Prothrombin                                      | 1145                          | 18                             | 6.65  | 0.78        | 6.78E - 04      |

<sup>a</sup>Fold change provided by MASCOT.

<sup>b</sup>P values calculated by edgeR to show the significance of different expression.
Table 4: Up- and downregulated serum proteins exclusively in DHSM-CHB.

| UniProt_ID          | Gene_name          | Description                                      | Number of significant matches | Number of significant sequences | emPAI | FC<sup>a</sup> | p value<sup>b</sup> |
|---------------------|--------------------|--------------------------------------------------|-------------------------------|--------------------------------|-------|--------------|-------------------|
| **Upregulated**     |                    |                                                  |                               |                                 |       |              |                   |
| A0A024R145.HUMAN    | ALDOB              | Fructose-bisphosphate aldolase                   | 5                             | 3                              | 0.22  | 1.29         | 3.81E – 02       |
| A0A024R1NL.HUMAN    | MYH9               | Myosin, heavy polypeptide 9, nonmuscle, isoform CRA.a | 17                            | 9                              | 0.13  | 1.43         | 1.34E – 03       |
| A0A024R5H8.HUMAN    | RAB6A              | RAS oncogene family, isoform CRA.b Actinin, alpha 1, isoform CRA.a | 8                             | 3                              | 0.57  | 1.43         | 4.84E – 03       |
| A0A024R694.HUMAN    | ACTN1              | Actinin, alpha 1, isoform CRA.a                   | 20                            | 10                             | 0.42  | 1.33         | 1.97E – 02       |
| A0A024R6G3.HUMAN    | FBLN5              | Fibulin 5, isoform CRA.b                         | 6                             | 3                              | 0.27  | 1.33         | 6.31E – 03       |
| A0A024R9T1.HUMAN    | hCG_39634          | HCG39634, isoform CRA.a                          | 2                             | 2                              | 0.26  | 1.49         | 4.90E – 03       |
| A0A024RDB8.HUMAN    | HPSE               | Heparanase, isoform CRA.a                        | 23                            | 6                              | 0.55  | 1.36         | 1.03E – 03       |
| A0A024RDL8.HUMAN    | ASL                | Argininosuccinate lyase isoform 1                | 10                            | 5                              | 0.39  | 1.65         | 2.02E – 07       |
| A0A087WT59.HUMAN    | TTR                | Transthyretin                                    | 175                           | 7                              | 5.66  | 1.34         | 3.42E – 02       |
| A0A087WU10.HUMAN    | TKFC               | Triokinase/FMN cyclase                          | 3                             | 3                              | 0.15  | 1.28         | 9.35E – 03       |
| A0A0A0MSD0.HUMAN    | SVEPI              | Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1 | 4                             | 3                              | 0.02  | 1.36         | 7.51E – 03       |
| A0A0A0MT32.HUMAN    | LIPA               | Lysosomal acid lipase/cholesteryl ester hydrolase | 2                             | 1                              | 0.09  | 1.38         | 4.78E – 03       |
| A0A0C4DFP6.HUMAN    | CRTAC1             | Cartilage acidic protein 1                      | 6                             | 5                              | 0.24  | 1.23         | 3.57E – 02       |
| A0A0S2Z3F6.HUMAN    | CETP               | Cholesteryl ester transfer protein plasma isoform 1 (fragment) | 95                            | 14                             | 3.56  | 1.36         | 1.42E – 03       |
| UniProt_ID | Gene_name       | Description                                                               | Number of significant matches | Number of significant sequences | emPAI | FC<sup>a</sup> | p value<sup>b</sup> |
|------------|----------------|--------------------------------------------------------------------------|------------------------------|--------------------------------|-------|--------------|-----------------|
| A0A125QYY9.HUMAN |            | IBM-B2 heavy chain variable region (fragment)                           | 12                           | 2                              | 0.83  | 1.42        | 2.25E-03         |
| A0N719.HUMAN | F5-20        | F5-20 (fragment)                                                          | 4                            | 1                              | 0.47  | 1.68        | 2.31E-03         |
| A2NYU9.HUMAN |            | Heavy chain Fab (fragment)                                               | 2                            | 1                              | 0.48  | 1.29        | 3.92E-02         |
| A8K486.HUMAN |            | Peptidyl-prolyl cis-trans isomerase                                       | 2                            | 2                              | 0.32  | 1.40        | 4.10E-02         |
| ADIPO_HUMAN | ADIPOQ       | Adiponectin                                                              | 27                           | 5                              | 2.13  | 1.32        | 1.12E-02         |
| APOF_HUMAN  | APOF         | Apolipoprotein F                                                         | 7                            | 3                              | 0.5   | 1.29        | 1.84E-02         |
| ASSY_HUMAN  | ASS1         | Argininosuccinate synthase                                               | 4                            | 3                              | 0.18  | 1.49        | 1.09E-04         |
| ATPB_HUMAN  | ATP5B        | ATP synthase subunit beta, mitochondrial cDNA FLJ38781 fis, clone LIVER2000216, highly similar to HEAT SHOCK COGNATE 71 kDa PROTEIN cDNA FLJ53743, highly similar to tubulin alpha-3 chain Proteasome (prosome, macropain) subunit, beta type, 2, isoform CRA_b | 11                           | 2                              | 0.22  | 1.34        | 3.74E-02         |
| B3KTV0_HUMAN |            |                                                                          | 5                            | 5                              | 0.21  | 1.29        | 3.41E-02         |
| B4DQK4.HUMAN |            |                                                                          | 21                           | 6                              | 0.81  | 1.61        | 1.06E-03         |
| B4DVA7.HUMAN |            |                                                                          | 3                            | 2                              | 0.15  | 1.26        | 1.84E-02         |
| B7Z478.HUMAN | PSMB2        |                                                                          | 2                            | 1                              | 0.3   | 1.65        | 1.96E-03         |
| BPIBL_HUMAN | BPIFB1       |                                                                          | 11                           | 6                              | 0.51  | 1.28        | 1.34E-02         |
| BTD_HUMAN   | BTD          |                                                                          | 47                           | 6                              | 1.12  | 1.54        | 1.69E-06         |
| CAMP_HUMAN  | CAMP         |                                                                          | 2                            | 1                              | 0.29  | 1.43        | 2.64E-03         |
| CAND1_HUMAN | CAND1        |                                                                          | 2                            | 1                              | 0.02  | 1.36        | 2.60E-03         |
| UniProt_ID     | Gene_name | Description                                      | Number of significant matches | Number of significant sequences | emPAI | FC<sup>a</sup> | p value<sup>b</sup> |
|---------------|-----------|--------------------------------------------------|-------------------------------|-------------------------------|-------|--------------|-------------------|
| CATD_HUMAN    | CTSD      | Cathepsin D                                      | 2                            | 1                             | 0.13  | 1.28        | 3.06E-02          |
| DHSO_HUMAN    | SORD      | Sorbitol dehydrogenase alpha chain               | 4                            | 3                             | 0.32  | 1.67        | 2.46E-07          |
| FIBA_HUMAN    | FGA       | Fibrinogen alpha chain                            | 102                          | 22                            | 2.36  | 1.36        | 3.18E-03          |
| G3P_HUMAN     | GAPDH     | Glycerol kinase 3-phosphate dehydrogenase        | 19                           | 7                             | 1.37  | 1.37        | 5.07E-03          |
| G3V5Z7_HUMAN  | PSMA6     | Proteasome subunit alpha type                     | 7                            | 4                             | 0.58  | 1.44        | 9.01E-04          |
| HV310_HUMAN   |           | Ig heavy chain V-III region HIL                   | 3                            | 1                             | 0.5   | 1.48        | 1.12E-03          |
| HV320_HUMAN   |           | Ig heavy chain V-III region GAL                   | 36                           | 3                             | 1.91  | 1.32        | 2.19E-02          |
| KV404_HUMAN   |           | Ig kappa chain V-IV region BI7 L-lactate dehydrogenase B chain | 34                           | 2                             | 1.45  | 1.45        | 4.98E-04          |
| LDHB_HUMAN    | LDHB      | L-lactate dehydrogenase B chain                   | 4                            | 2                             | 0.33  | 1.65        | 2.13E-06          |
| M0QZB5_HUMAN  | PPFIA4    | Liprin-alpha-4 (fragment)                         | 2                            | 1                             | 0.03  | 1.33        | 2.70E-02          |
| MARCO_HUMAN   | MARCO     | Macrophage receptor MARCO                        | 2                            | 1                             | 0.11  | 1.31        | 2.89E-02          |
| MDR3_HUMAN    | ABCB4     | Phosphatidylcholine translocator ABCB4            | 2                            | 1                             | 0.04  | 1.32        | 1.23E-02          |
| OTUB1_HUMAN   | OTUB1     | Ubiquitin thioesterase OTUB1 Polymeric immunoglobulin receptor | 2                            | 1                             | 0.09  | 1.27        | 2.92E-02          |
| PIGR_HUMAN    | PIGR      | Immunoglobulin receptor                           | 7                            | 4                             | 0.18  | 1.34        | 4.27E-03          |
| PSA3_HUMAN    | PSMA3     | Proteasome subunit alpha type-3                   | 8                            | 4                             | 0.58  | 1.31        | 4.84E-02          |
| PSBL_HUMAN    | PSMB1     | Proteasome subunit beta type-1                    | 4                            | 3                             | 0.5   | 1.47        | 1.25E-03          |
| Q6GMX4_HUMAN  | IGL@      | IGL@ protein                                     | 442                          | 6                             | 7.15  | 1.27        | 3.98E-02          |
Table 4: Continued.

| UniProt ID | Gene name | Description                                                                 | Number of significant matches | Number of significant sequences | emPAI | FC<sup>a</sup> | p value<sup>b</sup> |
|------------|-----------|------------------------------------------------------------------------------|-------------------------------|--------------------------------|-------|--------------|-------------------|
| Q6PIQ7_HUMAN | IGL@      | IGL@ protein                                                                 | 441                           | 6                              | 6.39  | 1.27         | 4.86E−02          |
| Q6ZNX5_HUMAN |           | CDNA FLJ26936 fis, clone RCT06808 Myosin-reactive immunoglobulin light chain variable region (fragment) | 5                             | 1                              | 0.39  | 1.78         | 2.10E−08          |
| Q9UL82_HUMAN |           |                                | 46                            | 3                              | 2.11  | 1.41         | 1.22E−03          |
| QSOX1_HUMAN | QSOXI     | Sulphhydryl oxidase 1                                                      | 97                            | 20                             | 2.33  | 1.24         | 4.15E−02          |
| TBA4A_HUMAN | TUBA4A    | Tubulin alpha-4A chain                                                      | 24                            | 8                              | 0.86  | 1.55         | 1.38E−03          |
| TBB1_HUMAN  | TUBB1     | Tubulin beta-1 chain                                                        | 6                             | 4                              | 0.33  | 1.30         | 2.87E−02          |
| TBB8_HUMAN  | TUBB8     | Tubulin beta-8 chain                                                        | 7                             | 5                              | 0.42  | 1.32         | 3.10E−02          |
| TRM1L_HUMAN | TREML1    | Trem-like transcript 1 protein                                              | 3                             | 1                              | 0.09  | 1.42         | 1.05E−03          |
| TSPL_HUMAN  | THBS1     | Thrombospondin-1                                                           | 365                           | 34                             | 4.23  | 1.37         | 1.66E−03          |
| TYPH_HUMAN  | TYMP      | Thymidine phosphorylase                                                     | 3                             | 3                              | 0.19  | 1.23         | 4.67E−02          |

**Downregulated**

| UniProt ID | Gene name | Description                                                                 | Number of significant matches | Number of significant sequences | emPAI | FC<sup>a</sup> | p value<sup>b</sup> |
|------------|-----------|------------------------------------------------------------------------------|-------------------------------|--------------------------------|-------|--------------|-------------------|
| A0A024CIM4_HUMAN |           | Carboxylic ester hydrolase Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 80, isoform CRA_b | 7                             | 3                              | 0.17  | 0.81         | 1.43E−02          |
| A0A024R6I6_HUMAN | SERPINAI0 |                                                                                | 65                            | 12                             | 2.73  | 0.75         | 1.46E−04          |
| A0A024R6K8_HUMAN | WARS     | Tryptophanyl-tRNA synthetase, isoform CRA_a                                    | 5                             | 2                              | 0.1   | 0.82         | 4.87E−02          |
| A0A024R853_HUMAN | IQCE     | IQ motif containing E, isoform CRA_b Collectin subfamily member 10 (C-type lectin), isoform CRA_a | 2                             | 1                              | 0.04  | 0.74         | 1.67E−03          |
| A0A024R9J3_HUMAN | COLEC10  |                                                                                | 4                             | 2                              | 0.28  | 0.83         | 2.40E−02          |
| A0A075B6S2_HUMAN | IGKV2D-29| Protein IGKV2D-29 (fragment)                                                  | 26                            | 3                              | 2.47  | 0.77         | 2.05E−03          |
| A0A087X054_HUMAN | HYOU1    | Hypoxia upregulated protein 1                                                 | 4                             | 2                              | 0.08  | 0.58         | 2.93E−08          |
| A0A0A7C3P2_HUMAN | HLA-A    | MHC class I antigen (fragment)                                                | 8                             | 3                              | 0.72  | 0.77         | 1.24E−03          |
| UniProtID          | Gene name              | Description                                                                 | Number of significant matches | Number of significant sequences | emPAI | FC<sup>a</sup> | p value<sup>b</sup> |
|-------------------|------------------------|-----------------------------------------------------------------------------|------------------------------|-------------------------------|-------|--------------|-------------------|
| A0A0J9YX35.HUMAN  | HUMAN Uncharacterized protein (fragment)                                 | 2                             | 1                             | 0.51                          | 0.74  | 3.43E-03     |                   |
| A0A0X9TD47.HUMAN  | HUMAN MS-D1 light chain variable region (fragment)                       | 30                            | 3                             | 5.49                          | 0.83  | 1.31E-02     |                   |
| A2AP.HUMAN        | SERPINF2                | Alpha-2-antiplasmin                                                         | 67                           | 10                            | 1.86  | 0.74         | 2.85E-04          |
| A2J1N4.HUMAN      | Rheumatoid factor RF-IP24 (fragment)                                     | 10                            | 2                             | 1.08                          | 0.80  | 4.59E-03     |                   |
| A2J1N5.HUMAN      | Rheumatoid factor RF-ET6 (fragment)                                      | 15                            | 1                             | 1.65                          | 0.79  | 6.40E-04     |                   |
| A2MYD0.HUMAN      | V1-17                   | V1-17 protein (fragment)                                                   | 8                            | 3                             | 1.87  | 0.83         | 1.21E-02          |
| A2NB45.HUMAN      | Cold agglutinin FS-1 L-chain (fragment)                                  | 23                            | 2                             | 1.39                          | 0.69  | 3.83E-04     |                   |
| A3RGK7.HUMAN      | Coagulation factor VII (fragment)                                        | 17                            | 6                             | 2.02                          | 0.77  | 7.55E-04     |                   |
| ALBU.HUMAN        | ALB                     | Serum albumin                                                              | 1841                         | 31                            | 17.82 | 0.67         | 1.64E-06          |
| AMBP.HUMAN        | AMBP                    | Protein AMBP                                                               | 34                           | 3                             | 0.75  | 0.76         | 4.96E-04          |
| ATSI3.HUMAN       | ADAMTS13                | ADAMTS13 protein, with thrombospondin motifs 13                            | 22                           | 8                             | 0.26  | 0.81         | 6.52E-03          |
| B2RBZ5.HUMAN      | Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 10 (SERPINA10), mRNA | 58                            | 11                            | 2.2                           | 0.76  | 1.77E-04     |                   |
| B4DL32.HUMAN      | Keratin, type II cytoskeletal 5 cDNA, FLJ59922, highly similar to Homo sapiens fibronectin 1 (FN1), transcript variant 4, mRNA | 7                             | 2                             | 0.33                          | 0.83  | 2.87E-02     |                   |
| B4DN2L.HUMAN      | Fibronectin 1 (FN1), transcript variant 4, mRNA                           | 384                           | 8                             | 6.85                          | 0.77  | 1.76E-03     |                   |
| UniProtID       | Gene name          | Description                                                                 | Number of significant matches | Number of significant sequences | emPAI | FC^a   | p value^b |
|----------------|--------------------|------------------------------------------------------------------------------|------------------------------|--------------------------------|-------|--------|-----------|
| B4E1B2_HUMAN   | HUMAN cDNAFLJ53691, highly similar to serotransferrin cDNA FLJ54395, highly similar to inter-alpha-trypsin inhibitor heavy chain H1 Brefeldin A-inhibited guanine nucleotide-exchange protein 3 | 142                           | 16                           | 2.78                          | 0.59  | 6.58E – 09 |
| B7Z8B6_HUMAN   | HUMAN cDNAFLJ54395, highly similar to inter-alpha-trypsin inhibitor heavy chain H1 | 68                            | 8                            | 1.2                           | 0.71  | 6.32E – 06 |
| BIG3_HUMAN     | ARFGEF3 Flavin reductase (NADPH) | 3                             | 1                            | 0.01                          | 0.71  | 7.55E – 03 |
| BLVRB_HUMAN    | BLVRB              |                                | 6                            | 2                             | 0.28  | 0.81  | 1.46E – 02 |
| CAT_HUMAN      | CAT catalase       |                                | 3                            | 3                             | 0.15  | 0.80  | 4.91E – 03 |
| CATE_HUMAN     | CTSF Cathepsin F   |                                | 6                            | 4                             | 0.23  | 0.81  | 2.58E – 03 |
| CO2_HUMAN      | C2 Complement C2   |                                | 20                           | 9                             | 0.5   | 0.78  | 1.25E – 03 |
| CO5_HUMAN      | C5 Complement C5   |                                | 416                          | 52                            | 4.64  | 0.74  | 4.50E – 04 |
| PI3B_HUMAN     | PI3B XIII B chain  |                                | 4                            | 3                             | 0.15  | 0.76  | 1.83E – 03 |
| GLUC_HUMAN     | GCG Glucagon       |                                | 2                            | 1                             | 0.3   | 0.81  | 8.52E – 03 |
| H0YL3_HUMAN    | B2M Beta-2-microglobulin (fragment) | 4                             | 1                            | 0.75                          | 0.77  | 5.98E – 03 |
| HEMO_HUMAN     | HPX Hemopexin      |                                | 63                           | 9                             | 1.51  | 0.65  | 4.59E – 08 |
| HGFA_HUMAN     | HGFAC Hepatocyte growth factor activator | 15                           | 4                            | 0.39                          | 0.82  | 4.99E – 03 |
| HV306_HUMAN    | Ig heavy chain V-III region BUT | 36                           | 5                            | 6.45                          | 0.76  | 2.60E – 04 |
| HV314_HUMAN    | Ig heavy chain V-III region LAY | 3                            | 2                            | 0.88                          | 0.81  | 8.62E – 03 |
| HV317_HUMAN    | Ig heavy chain V-III region ZAP | 3                            | 1                            | 0.56                          | 0.78  | 1.26E – 03 |
| ICL_HUMAN      | SERPING1 Plasma protease C1 inhibitor | 34                           | 7                            | 0.82                          | 0.62  | 1.32E – 09 |
| INHBC_HUMAN    | INHBC Inhibin beta C chain Inter-alpha-trypsin inhibitor heavy chain H1 | 16                           | 3                            | 0.36                          | 0.82  | 1.47E – 02 |
| ITIH1_HUMAN    | ITIH1              |                                | 252                          | 22                            | 3     | 0.81  | 1.20E – 02 |
| KIG0_HUMAN     | KRT10 Keratin, type I cytoskeletal 10 | 32                           | 8                            | 0.87                          | 0.70  | 1.75E – 06 |
Table 4: Continued.

| UniProtID       | Gene_name  | Description                          | Number of significant matches | Number of significant sequences | emPAI | FC^a | p value^b |
|-----------------|------------|--------------------------------------|-------------------------------|--------------------------------|-------|------|-----------|
| K1C14_HUMAN     | KRT14      | Keratin, type I cytoskeletal 14      | 3                             | 2                              | 0.11  | 0.68 | 1.74E - 06 |
| K1C9_HUMAN      | KRT9       | Keratin, type I cytoskeletal 9       | 24                            | 8                              | 0.65  | 0.81 | 5.29E - 03 |
| K22E_HUMAN      | KRT2       | Keratin, type II cytoskeletal 2      | 38                            | 14                             | 1.31  | 0.77 | 4.04E - 04 |
| K2C1_HUMAN      | KRT1       | Keratin, type II cytoskeletal 1      | 104                           | 19                             | 4.31  | 0.66 | 1.81E - 08 |
| K2C6B_HUMAN     | KRT6B      | Keratin, type II cytoskeletal 6B     | 18                            | 4                              | 0.51  | 0.63 | 3.76E - 09 |
| K7ER74_HUMAN    | APOC4-APOC2|                                      | 111                           | 5                              | 7.56  | 0.68 | 7.94E - 05 |
| KV201_HUMAN     | TRIM25     | Alternative protein TRIM25           | 13                            | 1                              | 0.9   | 0.77 | 8.84E - 04 |
| L0R8K6_HUMAN    | MASP1      | Mannan-binding lectin serine protease 1 | 36                        | 7                              | 0.63  | 0.83 | 1.78E - 02 |
| PCDGG_HUMAN     | PCDHGB4    | Protocaderhin gamma-B4               | 3                             | 1                              | 0.03  | 0.80 | 4.10E - 02 |
| PLAIA_HUMAN     | PLAIA      | Phospholipase A1 member A            | 2                             | 1                              | 0.07  | 0.83 | 5.39E - 03 |
| PROC_HUMAN      | PROC       | Vitamin K-dependent protein C        | 84                            | 9                              | 1.71  | 0.78 | 1.40E - 03 |
| Q06AH7_HUMAN    | TF         | Transferrin                          | 141                           | 16                             | 2.64  | 0.59 | 6.40E - 09 |
| Q1T720_HUMAN    | HLA-B      | MHC class I antigen (fragment)       | 9                             | 6                              | 0.9   | 0.77 | 1.30E - 03 |
| Q6U2L6_HUMAN    | C4B        | C4B (fragment)                       | 79                            | 6                              | 27.02 | 0.69 | 2.60E - 05 |
| Q9PIC5_HUMAN    | C4B        | PRO2769                              | 43                            | 7                              | 0.97  | 0.70 | 1.53E - 06 |
| Q9UMV1_HUMAN    | C4B        | Complement C4Bla (fragment)          | 8                             | 1                              | 1.56  | 0.77 | 3.10E - 04 |
| Q9UNU2_HUMAN    | C4B        | Complement protein C4B frameshift    | 524                           | 14                             | 26.83 | 0.64 | 8.08E - 07 |
| QPCT_HUMAN      | QPCT       | Glutaminyl-peptide cyclotransferase  | 7                             | 4                              | 0.43  | 0.83 | 1.22E - 02 |
| TIMP3_HUMAN     | TIMP3      | Metalloproteinase inhibitor 3        | 2                             | 1                              | 0.11  | 0.75 | 1.01E - 03 |

^a Fold change provided by MASCOT.

^b p values calculated by edger to show the significance of different expression.
are identified to interact with HBx as well [63]. Among the apolipoproteins APOA2 is a considerable biomarker because its expression is increased on both mRNA and protein levels in CHB patients [14, 64]. HBx protein also interacts with heat shock proteins and enhances HBx-mediated apoptosis [65]. A HBV-specific peptide (TVATAMG) is associated with heat shock protein and has potential for engineering tumor vaccines against hepatocellular carcinoma and chronic HBV infection [66]. Heat shock proteins like HSP27, HSP90, and GRP78 are upregulated in HBV related hepatocellular carcinoma, associated with vascular invasion and intrahepatic metastasis and have potential to be prognosis markers [67, 68]. Commonly downregulated complement proteins are important mediators of inflammation and contribute to the regulation of the immune response. C4, a predisposing factor to autoimmune chronic active hepatitis [69], is expressed lowly in chronic hepatitis C patient compared to that in controls [70]. Low serum levels of complement in viral hepatitis are associated with high titers of hepatitis-associated antigen [71]. It is said that complement proteins are related to hepatitis B vaccine and C4AQ0 (mutant C4) probably contribute to inefficient complement activation and failure of B cells to secret anti-HBs [72]. Our results confirmed the potential of previously reported proteins in diagnosis of patients infected by HBV.

LSSD-CHB and DHSM-CHB are two subtypes of CHB according to traditional Chinese medicine pattern classification. In this study we identified 47 and 119 differentially expressed proteins exclusively in LSSD-CHB and DHSM-CHB, respectively, which could be used as biomarkers for LSSD-CHB and DHSM-CHB patients. We showed top 5 highly expressed proteins with different expression in LSSD-CHB and DHSM-CHB patients compared to HCTL group in Figure 6. Using relative expression ratio calculated by MASCOT we found mean expression levels of some proteins were close in LSSD-CHB and DHSM-CHB but with different p values, such as CFH (complement factor H), F2 (prothrombin), and FGA (fibrinogen alpha chain). As we know, prothrombin time is one of the markers of liver test; it is usually lower in HBV infected patients than in healthy people and a good marker for liver fibrosis [73, 74]. CFH functions as a cofactor in the inactivation of C3b by factor I [75], which can
interact with IgG and is moderately depressed in the serum of patients with viral hepatitis [71]. FGA has a major function in hemostasis as one of the primary components of blood clots [76]. Fibrinogen-like protein 2 (FGL2) has been identified as a potential biomarker for severity of CHC infection [77]. Other proteins also have been reported to be associated with HBV infection. LGALS3BP (lectin galactoside-binding soluble 3 binding protein isoform 1) were downregulated in LSSD-CHB patients (fc = 0.71, \( p \) value = 2.49 \( E \) 06) and DHSM-CHB patients (fc = 0.89, \( p \) value = 0.061). Previous studies about LGALS3BP in CHB and HCC found its different expression on transcriptional level [78], while in current study we identified its protein was differentially expressed in CHB patients and had the potential to be a good marker for LSSD-CHB subtype. PON3 (serum paraoxonase/lactonase 3), which was upregulated exclusively in LSSD-CHB, might play a hepatoprotective role against histological alterations and hepatic cell apoptosis leading to liver disease [48].

DHSM-CHB specifically differentially expressed proteins like ITIH1 (inter-alpha-trypsin inhibitor heavy chain H1), THBS1 (thrombospondin-1), C5 (Complement C5), and ALB (albumin) have been also reported in hepatitis viral related diseases. The expression level of ITIH1 in HCTL group was similar to that in LSSD-CHB patients (fc = 0.99, \( p \) value =
0.227) but was downregulated significantly in DHSM-CHB patients (fc = 0.81, p value = 0.012). The low expression of ITIH indicated it can be used to differ DHSM-CHB from LSSD-CHB. In addition, it has been experimented to be downregulated in HCV infected patients [79] and hepatitis C associated hepatocellular carcinoma patients [80]. It is reported that HCV viral proteins act directly or indirectly on THBS1 in TGF-β pathway [81]. By noninvasive imaging the gene expression of THBS1 was upregulated in liver cancer [82]. Interestingly, ALB has been reported as an important factor to score the risk of HCC in CHB patients [83]. In our study, ALB was downregulated in both CHB subtypes but significantly exclusively in DHSM-CHB. Our results confirmed its different expression in CHB patients and revealed that the criteria of ALB expression in CHB patients required more patients and experiments. Due to the fact that hepatitis B viral load in DHSM-CHB patients was significantly higher than that in LSSD-CHB patients, we assume HBV-DNA might be related to CHB patients with different syndromes and it requires further experiments. To our knowledge, this study appears to be the first iTRAQ based approach aimed at identifying leads for potential useful biomarkers of patients of CHB subtypes. The candidates identified in this study await rigorous clinical validation using large cohorts of patient samples and more experimental function analysis.

Competing Interests

The authors have declared that no competing interests exist. And Zhenhua Zhuang and Bin Yang on behalf of Chengdu Life Baseline Technology declare there are no competing interests.

Authors’ Contributions

Jiankun Yang, Lichao Yang, and Quansheng Feng conceived and designed the experiments. Lichao Yang, Weilong Zhou, Sen Zhong, and Baixue Li performed the experiments. Jiankun Yang, Lichao Yang, and Maoshan Chen analyzed the data. Zhenhua Zhuang and Bin Yang contributed reagents/materials/analysis tools. Jiankun Yang, Lichao Yang, and Quansheng Feng wrote the paper. Jiankun Yang and Lichao Yang contributed equally to this work.

Acknowledgments

This study was supported by National Science and Technology Major Project of China (no. 2012ZX10005001-001).

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