Plasma biomarker screening for liver fibrosis with the N-terminal isotope tagging strategy

LI ShuLong[1,2†], LIU Xin[1,6†], WEI Lai[3], WANG HuiFen[4], ZHANG JiYang[1], WEI HanDong[1], QIAN XiaoHong[1*], JIANG Ying[1*] & HE FuChu[1,5*]

[1]State Key Laboratory of Proteomics, Beijing Proteome Research Center, Beijing Institute of Radiation Medicine, Beijing 102206, China;
[2]Beijing Institute of Biotechnology, Beijing 100071, China;
[3]People's Hospital of Peking University, Beijing 100044, China;
[4]302 Military Hospital of China, Beijing 100039, China;
[5]Institutes of Biomedical Sciences, Fudan University, Shanghai 200032, China;
[6]Central Laboratory, Yantai Yu-Huang-Ding Hospital, Yantai 264000, China

A non-invasive diagnostic approach is crucial for the evaluation of severity of liver disease, treatment decisions, and assessing drug efficacy. This study evaluated plasma proteomic profiling via an N-terminal isotope tagging strategy coupled with liquid chromatography/Fourier transform ion cyclotron resonance mass spectrometry measurement to detect liver fibrosis staging. Pooled plasma from different liver fibrosis stages, which were assessed in advance by the current gold-standard of liver biopsy, was quantitatively analyzed. A total of 72 plasma proteins were found to be dysregulated during the fibrogenesis process, and this finding constituted a valuable candidate plasma biomarker bank for follow-up analysis. Validation results of fibronectin by Western blotting reconfirmed the mass-based data. Ingenuity Pathways Analysis showed four types of metabolic networks for the functional effect of liver fibrosis disease in chronic hepatitis B patients. Consequently, quantitative proteomics via the N-terminal acetyl isotope labeling technique provides an effective and useful tool for screening plasma candidate biomarkers for liver fibrosis. We quantitatively monitored the fibrogenesis process in CHB patients. We discovered many new valuable candidate biomarkers for the diagnosis of liver fibrosis and also partly identified the mechanism involved in liver fibrosis disease. These results provide a clearer understanding of liver fibrosis pathophysiology and will also hopefully lead to improvement of clinical diagnosis and treatment.

quantitative proteomics, liver fibrosis, biomarker, plasma, hepatitis B virus

Hepatitis B virus (HBV) infection affects 350 million individuals worldwide [1] and more than 100 million individuals in China. Liver fibrosis is a common response to CHB infection and might result in potentially lethal sequelae, which progress to cirrhosis in 12%–20% of patients, and

compensated cirrhosis progresses to hepatic decompensation and hepatocellular carcinoma within five years in 20%–25% and 6%–16% of patients, respectively. Approximately 250000 deaths occur each year worldwide as a consequence of fulminant hepatic failure, cirrhosis and hepatocellular carcinoma [1,2]. Liver disease is not an incurable disease, because liver fibrosis and cirrhosis are reversible, and accurate diagnosis is crucial for evaluation of the sever-

ity of the disease, for treatment decisions, and for assessing drug efficacy [3]. Clinically, it is always difficult to make a noninvasive diagnosis of liver fibrosis. Liver biopsy is currently the gold-standard procedure for assessing the degree of fibrosis, but it has several disadvantages such as costly procedures, poor patient compliance, discomfort for the patient, the risk of poorly standardized collection of liver tissues, and limited usefulness for dynamic surveillance and follow-up. Considering these limitations, accurate and reliable noninvasive methods to assess the severity of hepatic fibrosis are urgently needed [4].

Plasma biomarkers offer an attractive alternative to liver biopsy because they contain considerable information on liver conditions and they are less invasive than biopsies, they may allow dynamic calibration of fibrosis, and may be more economic. Several markers and marker sets have shown promise towards this goal [5], but none of them fully satisfies the above requirements, especially for accurate staging of liver fibrosis in chronic hepatitis B (CHB) patients. Therefore, more suitable plasma markers with a high specificity and good sensitivity are required for non-invasive monitoring of liver fibrosis and its progression.

Proteomics is used to determine the expression and dynamics of many proteins, and is especially suitable for exploration of disease biomarkers. Comparison of proteomes of disease and control plasma samples has been shown to be a suitable approach for discovering plasma biomarkers of liver diseases [6]. Quantitative proteomic techniques based on stable isotope labeling are increasingly being applied in biomarker studies [7,8]. The N-terminal acetyl isotope (d0/d6) labeling technique shows great potential in biomarker detection with its high-throughput identification and accurate quantification of proteins, as well as being a simple procedure. Leading edge liquid chromatography/Fourier transform ion cyclotron resonance mass spectrometry (LC-FTICR-MS) technology's superior sensitivity and powerful measurement can also contribute to plasma biomarker detection. Therefore, this technique was selected as the strategy for our study [9].

Based on the histology of chronic liver diseases, this study was designed to explore candidate plasma biomarkers that are correlated with the stages of pathology with the quantitative proteomics technique, N-terminal acetyl labeling. We aimed to investigate new candidate plasma biomarkers for the accurate and noninvasive diagnosis of liver fibrosis in CHB patients to improve staging of liver disease and to assess the effect of treatment and predict disease progression.

1   Materials and methods

1.1   Patient selection

Liver biopsies were obtained from consecutive CHB pa-

tients in the People's Hospital of Peking University and 302 Military Hospital of China. The study was approved by the local ethics committee and written informed consent was obtained for all participants. Clinical, hematological, and biochemical data were recorded from each patient within one week of liver biopsy. The included patients all had serum positive for hepatitis B surface antigen, hepatitis B e-antigen, and hepatitis B virus DNA for at least six months. Serum levels of alanine aminotransferase were between two and 10 times the upper limit of normal within six months before liver biopsy and there was no prior therapy with antiviral or immunoregulating drugs. Those patients who had positive laboratory tests for human immunodeficiency virus, positive serum for anti-hepatitis C virus (anti-HCV), and/or HCV RNA, decompensated liver disease, alcoholic liver disease, suspected autoimmune diseases with an antinuclear antibody titer greater than 1:160, serum creatinine 1.5 times greater than normal, and inadequate hematological function, were all excluded.

1.2   Histological examination

All selected patients received a liver biopsy directed by ultrasonography within one week after inclusion. A quick-cut (8-Light Company, Japan) or Menghini needle (14 G) was used for this procedure. The biopsy specimens were fixed with 10% formalin, routinely embedded in paraffin, and the tissue sections were processed with hematoxylin and eosin, Masson's trichrome, and reticular fiber staining (Appendix Figure S1 in the electronic version). A minimum length of at least 1 cm of the liver biopsy and at least six portal tracts were required for diagnosis. Five fibrosis stages were determined using Scheuer's classification [10]: stage 0 (F0), no fibrosis; stage 1 (F1), enlarged fibrotic portal tracts; stage 2 (F2), periportal or portal-portal septa, but intact architecture; stage 3 (F3), fibrosis with architectural distortion, but no obvious cirrhosis; and stage 4 (F4), cirrhosis. Liver fibrosis was considered significant when it spread beyond the portal tract (stages 2, 3, or 4), whereas it was considered insignificant when it was absent or restricted to the portal tract (stages 0 or 1, respectively). All the sections were blindly and independently assessed by two pathologists from the above hospitals.

Detailed information for patient characteristics such as sex, age and type of disease is shown in Table 1 and Appendix Table S1 in the electronic version.

1.3   Plasma treatment

After obtaining informed consent, we collected 5 mL fasting blood samples by venipuncture and a BD Vacutainer K3 EDTA (BD Biosciences, Franklin Lakes, NJ, USA). Plasma was obtained by centrifuging the blood at 2000×*g* for 15 min at 4°C, and then supernatant was transferred to fresh microtubes (Axygen Scientific, Inc., USA). They were cen-

Table 1   Characteristics of CHB patients with different liver fibrosis stages[a)]

Fibrosis stage	F0	F1	F2	F3	F4
Age (SD)	32 (10.6)	30.2 (13.5)	31.5 (8.3)	37.5 (10.2)	36.4 (11.9)
Sex (F/M)	4/6	4/6	4/6	3/6	3/7
Race (Han)	10	10	10	9	10
Fibrosis activity	G0	G2	G2	G2	G2
HCV	0	0	0	0	0
HIV	0	0	0	0	0
Alcoholic liver disease	0	0	0	0	0
Autoimmune diseases	0	0	0	0	0

a) F0–F4 refer to stage 0–4 for liver fibrosis, respectively. The individual detailed information was attached in Appendix Table S1 in the electronic version.

trifuged at 15000×*g* for 15 min at 4°C and stored at −70°C before analysis. All patients had been fasting for at least 6 h before blood sampling. To remove albumin and immunoglobulin from human plasma, the samples were treated with ProteoExtract™ Removal Kits (Merck Company, Germany) according to the manufacturer's instructions (Appendix Figure S2 in the electronic version). The treated samples were measured at 595 nm for protein concentration with a Protein DC kit (Bio-Rad, USA) based on the Lowry method. To evaluate the reproducibility and effect of removal, all the treated samples were separated with 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), 30 μg protein per lane, and stained by SYPRO-Ruby (Bio-Rad). The main procedure of this research is shown in Figure 1.

1.4   Plasma pooling and SDS-PAGE fractionation

All the individual plasma included was measured for protein concentration with a Protein DC kit (Bio-Rad), and then all the individual plasma in the same group was pooled equally before the following fractionation, which represents the corresponding fibrosis stage (F0–F4). All the plasma from the five groups was pooled equally as the intra-standard (*P*). Each group (F0–F4, pooled and treated plasma) was compared with the same intra-standard (*P*), and the corresponding ratio value was used for their corresponding protein's quantified data. A total of 50 μg treated plasma protein from each group (P, F0–F4) was boiled in SDS-PAGE sample buffer, resolved on a 12% SDS-PAGE gel and stained with Coomassie Blue. Each group in a gel lane with its neighboring intra-standard gel lane was excised in a parallel manner, and the gel was divided horizontally into 15 sections in total and processed separately for tryptic digestion as described below (Appendix Figure S3 in the electronic version).

1.5   In-gel tryptic digestion

The gel particles were washed twice with a suitable volume (to immerse the gel particles) of 25 mmol L^{-1} ammonium
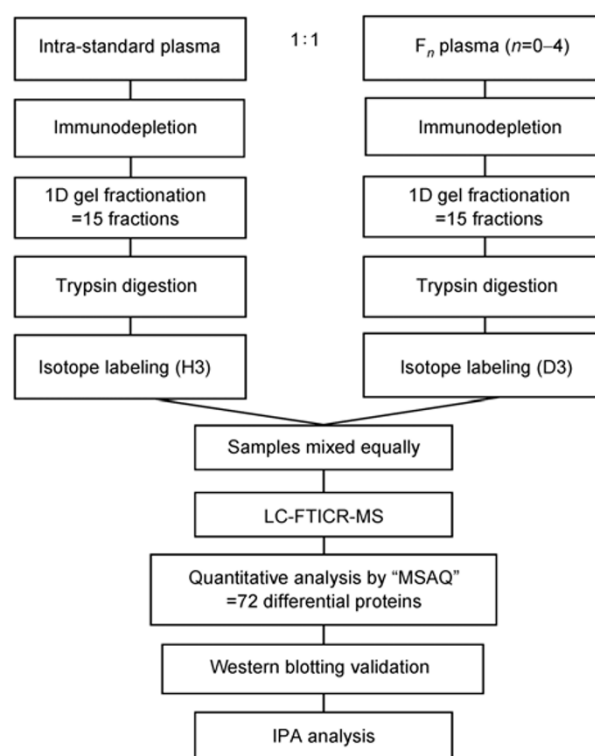
Figure 1   Schematic diagram of human plasma quantitative proteome analysis using the N-terminal acetyl labeling approach. Pools of all the plasma samples from F0 to F4 along with the same intra-standard (intra standard plasma, pool of all the plasma included in this study) were similarly processed and fractionated by SDS-PAGE and combined after differential isotopic labeling. Individual fractions were analyzed by LC-MS/MS. Data were processed using "MSAQ".

bicarbonate/50% acetonitrile (Sigma, USA). The supernatant was removed and discarded after each wash. The destained gel particles were dried for 30 min in a Speed-Vac (Thermo Electron, USA), and then rehydrated in 10 μL 25 mmol L^{-1} ammonium bicarbonate buffer containing 0.1 μg μL^{-1} trypsin at 4°C for 30 min. The gels were overlaid with 25 mmol L^{-1} ammonium bicarbonate buffer and incubated 12–16 h at 37°C. The peptides were extracted by 5% trifluoroacetic acid (TFA) and then by 50% acetonitrile/5% TFA. The supernatant was removed after each step and

saved in tubes. The peptides were dried in the Speed-Vac and stored at −80°C.

1.6 N-terminal isotope labeling

The ε-amino group of all lysine was guanidinated as described previously [11,12] with some modifications. An equal volume of 1 mol L^{-1} O-methylisourea (dissolved in 0.5 mol L^{-1} carbonate buffer, adjusted to pH 11 with 1 mol L^{-1} NaOH) was added to the peptide mixture and incubated at 37°C for 2 h. The solutions were adjusted back to pH 8.0 with 1 mol L^{-1} HCl for acetylation. A total of 200 μL of borate buffer was added to the solution for maintenance of the pH value. D0- and d6-acetic anhydrides were diluted into 1 mol L^{-1} with tetrahydrofuran, and then they were added to the guanidinated peptides. The amount of diluted acetic anhydride used was 1 μL per 50 μg digested peptides. The acetylation reaction was performed at room temperature for 1 h. N-hydroxylamine was used to hydrolyze the esters formed during the acetylation reaction by incubation for 30 min (pH 11.0). The resultant solutions were then desalted on a C18 column, lyophilized, and stored at −20°C before use.

1.7 Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis

The resultant plasma peptides were analyzed using a 7-Tesla linear quadrupole ion-trap Fourier-transform ion-cyclotron resonance (LTQ-FT) mass spectrometer (Thermo Electron, San Jose, CA, USA) coupled with an Agilent 1100 nano-flow liquid chromatography system. Reverse phase C18 trap columns (300 μm internal diameter×5 mm long column) were connected with a six-port column-switching valve for on-line desalting. A PicoFrit™ tip column (Bio-Basic C18, 5 μm particle size, 75 μm internal diameter×10 cm long column, 15 μm internal diameter at the spray tip; New Objective, Woburn, MA, USA) was used for the following separation. Elution was performed with solvent A (Milli-Q water, 2% acetonitrile and 0.1%TFA, v/v/v) and solvent B (Milli-Q water, 80% acetonitrile and 0.1%TFA, v/v/v). The gradient was 15%–40% B for 80 min and 40%–100% B for 15 min. One FT full MS scan was followed by five data-dependent LTQ MS/MS scans on the five most intense ions. The dynamic exclusion time was 45 s. Ions were accumulated in a linear ion trap controlled by the automatic gain control. The automatic gain control values were 5×105 charges for the FT full MS scan and 1×104 charges for the LTQ MS/MS scan. The resolution was 10000 for a FT full MS scan at 400 *m/z*. The temperature of the ion transfer tube was set at 200°C and the spray voltage was set at 1.8 kV. The isolation width was 4 Da and normalized collision energy was 35% for the MS/MS scan. Mass spectra were acquired over the mass range from 400 to 2000 *m/z*.

1.8 Protein identification and quantification

The acquired raw file was converted to *.dta files by Bio-Works 3.2 and all the *.dta files were merged into one *.mgf format file by a script named merge.pl. The *.mgf file was then searched against the international protein index (IPI) Human v3.07 (ftp://ftp.ebi.ac.uk/pub/databases/IPI/old/HUMAN/ipi.HUMAN.v3.07.fasta.gz) by the in-house MASCOT (version 1.9). The d0-acetylation (+42.0106 Da) and d3-acetylation (+45.0294 Da) of the peptide N-terminus, guanidination of the lysine residue (+42.0218 Da), and cysteine carbamidomethylation (+57.0214 Da) were set as fixed modifications. The maximal allowable miss-cleavage sites were two, and the mass tolerances for peptide and fragment ions were $1.5×10^{-5}$ and 1.0 Da, respectively. There was at least 95% confidence that each identified peptide had a score greater than 27. Each identified protein had at least one unique positive peptide that was not shared with any other protein. Based on the mascot results, the monoisotopic peak ratio between the heavy-labeled peptides and the light-labeled peptides was automatically calculated by a graphic user interface program called "mass spectrometry quantification with acetylation labeling" (MSAQ) [13] developed in house. The basic quantitative information was from the intensities of the pair of parent ions of the light- or heavy-labeled peptides in the MS full scans. The peptide pairs were accurately extracted by matching the *m/z* of the parent ion with the peaks in the MS full scan around the MS/MS scan time, and the intensity ratio of the labeled peptide pairs was computed. Because the 4th isotopic peak of the light-labeled peptide might overlap with the monoisotopic peak of the heavy-labeled peptide, especially when the mass is greater than 1500 Da, the observed ratio could be larger than the true ratio. According to the theory that isotopic distribution can be calculated by a polynomial algorithm, the overlapping portion was subtracted. After the steps mentioned above, the mean and standard deviation of the ratios of the pairs of each peptide were calculated and used as the final quantitative ratio of the peptide. To maintain data quality, the minimal signal/noise (S/N) of the pairs was set to greater than two, which was read from the *.raw file by the Xcalibur Development Kit (XDK) API function.

1.9 Ingenuity pathway analysis (IPA)

Predominant interaction networks of differentially expressed proteins were conducted using IPA on a web-delivered application (www.ingenuity.com) [14]. The biological functions assigned to each network were ranked according to the significance of that biological function to the network. Fischer's exact test was used to calculate the *P*-value.

1.10 Western blotting verification

Samples were run on 12% SDS-PAGE gels and transferred

onto polyvinylidene difluoride membranes in a trans-blot electrophoresis transfer cell (Bio-Rad). The membranes were blocked overnight at 4°C in 20 mmol L^{-1} Tris-HCl, 140 mmol L^{-1} NaCl, pH 7.5, and 0.05% Tween-20 (TBST) containing 5% skim milk. The primary antibody used was fibronectin antibody (diluted 1:1000, Santa Cruz Biotechnology, USA). Membranes were incubated at room temperature for 1 h with each primary antibody, and then they were washed three times with 0.05% Tween-20 and incubated with horseradish peroxidase-conjugated secondary antibody (diluted 1:10000, Santa Cruz Biotechnology) for 1 h at room temperature. Visualization of the immunoreactive proteins was accomplished using enhanced chemiluminescence reagents (Santa Cruz Biotechnology). All the membranes were exposed on the same X-ray film and scanned by a GS-710 scanner (Bio-Rad).

2 Results and discussion

2.1 Results

We quantified a total of 1109 plasma proteins, among which 72 proteins (ratio (max/min)$\geqslant$1.5, quantified peptides$\geqslant$2, expression in all liver fibrosis stages) were dysregulated in the liver fibrosis stages (F0–F4), and this provided a valuable reference and biomarker bank for liver fibrosis detection. The data are listed in Appendix Tables S2 and S3 in the electronic version. Some mass spectra for selected peptides are shown in Figure 2. Other mass spectra for selected peptides are attached in Appendix Figure S4 in the electronic version.

Detailed information with all quantitative data is listed in Appendix Table S3.

2.2 Western blot verification

To validate reliability of the NIT (N-terminal isotope tagging) results, one of the identified proteins, fibronectin, was selected for further examination by Western blotting. This protein has been reported in HCV patients, but there are no reports in CHB patients, especially for liver fibrosis stages. We showed a similar expression to the data from the NIT proteomic technique, which re-confirmed the proteomic data as well as their potential clinical use (Figure 3).

2.3 Discussion

The search for disease-specific biomarkers from human plasma is gaining increasing attention because of significant advances in proteomic technology and their potential for discovering novel disease biomarkers [15]. Noninvasive plasma biomarker diagnosis for hepatic fibrosis has become popular because it provides considerable information, which is crucial for evaluation of the severity of the disease, for treatment decisions, and for assessing drug efficacy. Currently, the available noninvasive methods of assessing liver fibrosis in CHB patients are imperfect and lack accuracy
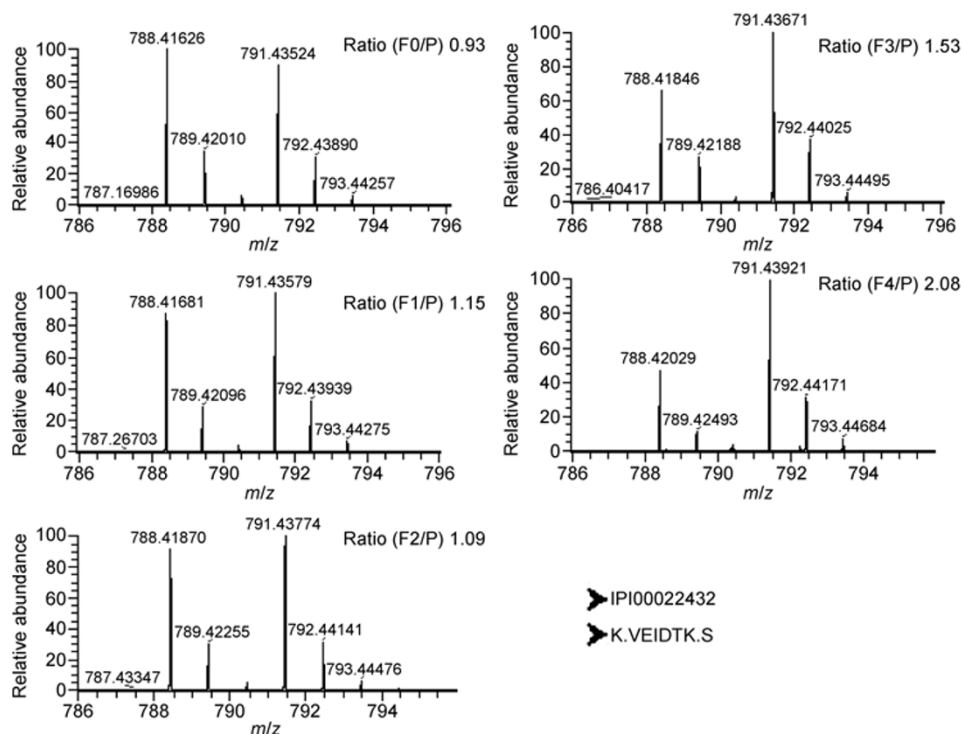
Figure 2   Mass spectra for a selected peptide of transthyretin with the ratios of the five different liver fibrosis stages. The peptide shows up-regulated expression with development of liver fibrosis, especially during the F3 and F4 stages.
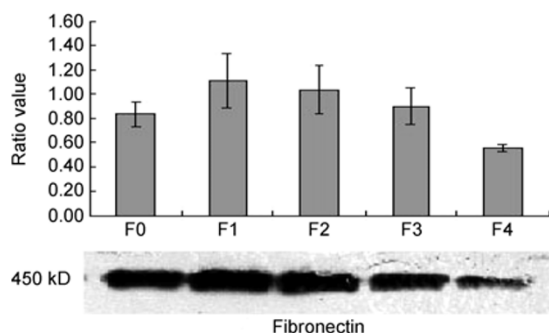
Figure 3 MS-based quantitative pattern and Western blotting result for fibronectin in the five different liver fibrosis stages (F0–F4). The fibronectin ratio value was obtained by MS-based quantitation of seven peptides, with the corresponding standard error indicated on the histogram. The results show satisfactory agreement with the Western blotting results.

and reliability [16,17]. Therefore, more reliable biomarkers are required to solve this issue.

2.3.1 NIT technology is reliable and effective for biomarkers' screening

Based on the high mass accuracy and sensitivity instrumentation of LTQ-FT, stable isotope labeling techniques produce reliable results in quantitative proteome analysis [18,19]. With its advantages of simple labeling procedures, low cost, full labeling ability for all the proteins, and a high accuracy and sensitivity, N-terminal isotope labeling technology is a reliable and powerful strategy for biomarker analysis [20,21]. We quantified a total of 1109 proteins. Based on the aim of stringently screening with a high confidence, we only chose proteins that were markedly dysregulated in all the five groups (F0–F4) and had at least two peptides quantified in the same protein. As a result, 72 different proteins were found in the quantitative analysis among all the liver fibrosis stages, and we were then able to construct a reliable dataset for detection of liver fibrosis.

2.3.2 A candidate biomarker dataset with new properties

Liver fibrosis is a dynamic and multi-factorial process involving complex interactions within many types of cell types. When this process is disrupted there is an accumulation of several distinct extracellular matrix (ECM) proteins [22]. Hepatitis B causes liver fibrosis through direct cytopathic means, direct and indirect interactions with hepatic stellate cells (HSCs), as well as activation of the immune system. Plasma proteins contain many messages for the fibrogenesis process both directly and indirectly. In this study, the significantly dysregulated proteins were related to several types of mechanisms, such as HSC dysfunction, immune factor impairment, lipid metabolism, protein synthesis in the liver, and hematological system development. Some of these candidate proteins are discussed below.

(i) Proteins related to HSC dysfunction. HSCs play a central role in the fibrogenesis process, and HSC dysfunction is reflected in several aspects. Gelsolin, which was one

of the candidate proteins for LF diagnosis in our study, is an actin-fragmenting cytoplasmic protein and it plays an important role in HSC growth regulation, and it is suggested to be involved in cell motility, to inhibit apoptosis and to have a protective role in liver tissue [23,24]. Gelsolin levels are decreased during liver injury or liver cancer [25]. We found that this protein had down-regulated expression from the F3 to F4 stages, which suggests that liver cell apoptosis was intensified and it indicates its potential value for clinical diagnosis.

Fibronectin is a glycoprotein, the major sources of which are hepatocytes, Kupffer cells and endothelial cells. It has many biological functions including adhesion between cells, immunity, blood coagulation and platelet aggregation. It has been reported to be down-regulated in liver cirrhosis in HCV patients [26,27]. However, its sensibility and accuracy for liver fibrosis staging, especially for CHB patients, is unknown. We found that fibronectin was down-regulated between the F3 and F4 stages, which shows its potential power for liver fibrosis staging.

(ii) Proteins related to liver synthesis and function. Protein synthesis is mostly carried out in the liver. Any damage to the liver, including liver fibrosis disease caused by HBV, will be reflected by plasma proteins.

Hemopexin is a plasma glycoprotein that is expressed only in the liver. It is synthesized at a lower rate in the fetal liver than in the adult [28–30]. Hemopexin is essential for protection from splenomegaly and liver fibrosis resulting from intravascular hemolysis. In our study, we observed down-regulated expression of hemopexin, which suggests that it has a dysfunctional role in liver fibrosis disease.

Vitronectin is a multifunctional plasma glycoprotein produced by hepatocytes. It has been extensively studied as a cell adhesion molecule and may play an important role in the progression of liver disease and/or in hepatic fibrosis through its collagen-binding domain [31,32]. We found that vitronectin was up-regulated in this study, which suggests that it could be a potential biomarker for fibrogenesis.

Orosomucoid (alpha1-acid glycoprotein) is a polymorphic acute-phase reactant with immunosuppressive properties and has been found to be significantly increased in patients with different types of carcinomas [33]. Previous investigations have suggested that orosomucoid and other acute-phase reactants may act as blocking factors protecting tumor cells against immunological attack, thereby contributing to the "immune escape" of the tumor. It is required to maintain the high capillary permselectivity required for normal homeostasis [34]. In our study, we observed that orosomucoid tended to be up-regulated with liver fibrosis development. This result should be helpful for detection of liver fibrosis as well for determining its molecular mechanism.

Amyloid P component is a plasma protein with a possible role in liver protein synthesis and it might be useful as a marker of liver involvement [35,36]. Amyloid P component

plasma levels and the degree of disease activity and hepatic impairment are closely correlated in patients with liver diseases. In the current study, amyloid P component expression was decreased in F4/F2. This result may be helpful for routine judgments.

Ficolin is synthesized in the liver, and it plays an important role in the lectin pathway of the complement system [37,38]. Its down-regulated expression following the fibrogenesis process potentially makes it a suitable biomarker in this study. We found that both isoforms of kininogen of this protein [39] were down-regulated in the F4 stage.

(iii) Proteins related to immune factors.   Chronic viral diseases of the liver are associated with changes in immune reactions mediated by immune effector mechanisms [40]. Immunoglobulin is one of the main proteins in immune responses and is expressed differently in different diseases. Recently, it was reported that it appeared to be correlated with the degree of fibrosis with different glycosylation [41]. In our study, we also observed several types of immunoglobulin proteins or peptides related to different liver fibrosis stages. Different expression of this protein may be related to the natural history of the disease and it may be useful in the noninvasive detection of fibrosis and cirrhosis. We found that the immune-related proteins, IGKV1-5/3-25/2 protein and complement factor C4-A, 4B and 5 all showed dysregulated expression during liver fibrosis development, which suggests that they play an important role participating an alternative pathway in HBV disease.

(iv) Proteins related to lipid metabolism.   Several types of apolipoprotein are clinically used as noninvasive biomarkers for liver fibrosis diagnosis in HCV patients, non-alcoholic fatty liver disease and carcinoma [42–44]. One type of pathway that affects disease development is apolipoprotein entering cells via the low-density lipoprotein receptor. We identified apolipoprotein groups including apolipoprotein A-IV, B, D, E, L, and M, which all showed dysregulated expression during the fibrogenesis process, which indicates that lipid metabolism plays an important role during fibrogenesis development in CHB patients.

(v) Proteins related to development of the hematological system.   Hemoglobin from erythrocytes is a source of iron that accumulates in the liver, and this mediates liver cell damage in viral hepatitis [45]. Using the SELDI (surface enhanced laser desorption ionization) system, hemoglobin was found to be correlated with liver fibrosis in CHB patients [46]. We found three types of subunits of hemoglobin, which could be used to differentiate between different stages of fibrosis in CHB patients.

The renin-angiotensin system regulates fibrosis [47]. As a principal effector molecule of the renin-angiotensin, angiotensinogen is mainly produced in the liver, and it played multi-roles in human HSCs and liver cirrhosis in patients with chronic hepatitis B [48]. In our study, we found that this protein's expression was up-regulated in the F2 stage compared with that in other liver fibrosis stages in CHB patients.

(vi) Proteins grouped as enzymes.   Several types of enzymes have been used as biomarkers for the diagnosis of liver disease [49–51]. Aldehyde dehydrogenase has previously been reported to play a pathogenetic role in the development of alcoholic liver disease [52,53]. In the current study, it was down-regulated in the F3 and F4 stages, suggesting a functional effect by hepatitis B disease as well as its potential for diagnosis.

Paraoxonase-1 is an esterase that degrades oxidized lipids, and it is associated with hepatic disease [54]. We found that paraoxonase-1 expression was up-regulated, which makes this protein a suitable candidate marker monitoring LF process.

(vii) Other candidate proteins.   Among the candidate proteins, several of them were dysregulated in the fibrogenesis process. Alpha-2-glycoprotein-1 is a plasma protein of unknown function, which has been reported as a biomarker for some diseases including microbial infections and cancer [55,56]. In this study, it was up-regulated in F3 stage, which would be a valuable candidate protein for LF diagnosis. Serpin peptidase inhibitor has been reported as a biomarker for several diseases [57,58]. A recent report also showed its biomarker potential in fibrosis progression in HCV patients [59]. Different types of serpin peptidase inhibitors (clade A, member 8, clade C&D, and F&G) in our study all showed increased expression in the F4 stage, which suggests their potential for liver fibrosis detection. Clusterin has been reported to be a biomarker for several diseases [60–62], and its overexpression occurs in various human malignancies [63]. In the current study, clusterin showed dysregulated expression, which should be useful information for liver fibrosis analysis. Transthyretin is primarily synthesized by the choroid plexus and can bind to beta-amyloid peptide. Several diseases have been associated with this protein [64,65]. It may also be a potential biomarker for the F1 and F4 stages in CHB patients.

It is necessary to mention that there are some proteins only with its molecular weight or CDNA code or hypothetical protein, but they also showed close correlation with LF stages in CHB patients. Further studies are required to validate their potential value in detail. It should also be noted that some of our identified proteins are different isoforms, truncated forms or post-translational modifications such as phosphorylation. Therefore, the coming test of verification for these proteins should also be carried out. Our study provides insight into possible compensatory mechanisms regarding the process of liver fibrosis as well as being a valuable reference for clinical diagnosis.

2.3.3   The IPA results suggest potential mechanisms of fibrogenesis

IPA is web-based software used for identifying the biological

mechanisms, pathways and functions most relevant to experimental datasets of interest (http://www.ingenuity.com). It allows profiling data to be analyzed in a systematic way using known pathways represented by the proteins undergoing change [66]. The identified proteins are mapped onto networks available in the Ingenuity Database. Using an uploaded file with 72 IPI proteins, four pathways were constructed by IPA (Appendix Figure S5 and Table S4 in the electronic version), which were related to cellular assembly and organization and development, development and function of the hepatic system, neurological disease, development and function of the hematological system, cardiac damage, cardiovascular disease, and skeletal and muscular disorders.

The pathogenesis of fibrosis and cirrhosis during chronic viral hepatitis is multi-factorial, involving recurrent liver injury, sustained inflammation, and differential activation of ECM-producing cells [67,68]. The liver is the main site for the synthesis and release of most of the plasma proteins, and when it is infected by HBV, plasma proteins are likely to be quantitatively and qualitatively affected. IPA analysis with these differentially expressed proteins could contribute to comprehension of the mechanism of fibrogenesis in CHB patients. Therefore, further studies are required on the proteins related to these pathways.

2.3.4 Possible patterns for diagnosis of liver fibrosis

There is a growing consensus that panels of markers should be able to supply the specificity and sensitivity that an individual marker lacks. An integrated test or fingerprinting with many differential proteins identified in this study may better reflect a patient's status than one marker alone. For example, haptoglobin, hemoglobin, and IGLC (immunoglobulin-free light chains) may be coupled together when LF diagnosis, which should be more reliable for liver fibrosis diagnosis. Many other proteins could also be detected in parallel in follow-up analysis. However, many candidate proteins found in this study have no commercially available kits for their detection. Therefore, protein-chip technology [69,70] or multiple reaction monitoring mass spectrometry technology [71] could possibly be performed simultaneously in a single experiment, to detect and quantify specific proteins or peptides in plasma of patients with liver fibrosis to determine the degree of hepatic fibrosis. A number of studies have shown the feasibility of such an approach [72].

3 Conclusion

Further study is required to validate the use of this model in CHB patients in a prospective manner with a greater number of valid plasma samples. Candidate biomarkers should also be tested in CHB patients on a large scale with suitable verification methods, such as enzyme-labeled immunosorbent assay or multiple reaction monitoring, to determine

their general applicability to fibrosis staging and, ultimately, for the potential of plasma proteomics as noninvasive markers of fibrosis and cirrhosis.

Overall, in our study, N-terminal isotope tagging strategy, which is a reliable, cost-effective and undemanding procedure in high-throughput proteomics experiments [21,73], showed existing and new candidate markers for fibrosis progression in CHB patients. These data constitute a valuable reference and provide new opportunities for better follow-up of HBV-infected patients, which link them mechanistically to the molecular events underlying liver fibrosis disease and detection. Ultimately, such biomarkers could aid clinicians in diagnosing development of liver fibrosis, eliminating the need for liver biopsy and allowing early treatment, and monitoring fibrosis development during therapy.

1 Lee W M. Hepatitis B virus infection. N Engl J Med, 1997, 337: 1733–1745
2 Lok A S, McMahon B J. Chronic hepatitis B. Hepatology, 2001, 34: 1225–1241
3 Afdhal N H, Nunes D. Evaluation of liver fibrosis: a concise review. Am J Gastroenterol, 2004, 99: 1160–1174
4 Friedman S L. Liver fibrosis—from bench to bedside. J Hepatol, 2003, 38: S38–53
5 Smith J O, Sterling R K. Systematic review: non-invasive methods of fibrosis analysis in chronic hepatitis C. Aliment Pharmacol Ther, 2009, 30: 557–576
6 Beretta L. Comparative analysis of the liver and plasma proteomes as a novel and powerful strategy for hepatocellular carcinoma biomarker discovery. Cancer Lett, 2009, 286: 134–139
7 Ong S E, Mann M. Mass spectrometry-based proteomics turns quantitative. Nat Chem Biol, 2005, 1: 252–262
8 Tao W A, Aebersold R. Advances in quantitative proteomics via stable isotope tagging and mass spectrometry. Curr Opin Biotechnol, 2003, 14: 110–118
9 Wu S, Kaiser N K, Meng D, *et al.* Increased protein identification capabilities through novel tandem MS calibration strategies. J Proteome Res, 2005, 4: 1434–1441
10 Scheuer P J. Classification of chronic viral hepatitis: a need for reassessment. J Hepatol, 1991, 13: 372–374
11 Beardsley R L, Reilly J P. Optimization of guanidination procedures for MALDI mass mapping. Anal Chem, 2002, 74: 1884–1890
12 Ji C, Guo N, Li L. Differential dimethyl labeling of N-termini of peptides after guanidination for proteome analysis. J Proteome Res, 2005, 4: 2099–2108
13 Jia N, Liu X, Wen J, *et al.* A proteomic method for analysis of CYP450s protein expression changes in carbon tetrachloride induced male rat liver microsomes. Toxicology, 2007, 237: 1–11
14 Meneses-Lorente G, Watt A, Salim K, *et al.* Identification of early

proteomic markers for hepatic steatosis. Chem Res Toxicol, 2006, 19: 986–998

15 Nedelkov D, Kiernan U A, Niederkofler E E, *et al*. Investigating diversity in human plasma proteins. Proc Natl Acad Sci USA, 2005, 102): 10852–10857

16 Lackner C, Struber G, Liegl B, *et al*. Comparison and validation of simple noninvasive tests for prediction of fibrosis in chronic hepatitis C. Hepatology, 2005, 41: 1376–1382

17 Rockey D C, Bissell D M. Noninvasive measures of liver fibrosis. Hepatology, 2006, 43: S113–120

18 Everley P A, Bakalarski C E, Elias J E, *et al*. Enhanced analysis of metastatic prostate cancer using stable isotopes and high mass accuracy instrumentation. J Proteome Res, 2006, 5: 1224–1231

19 Koumenis I L, Vestal M L, Yergey A L, *et al*. Quantitation of metal isotope ratios by laser desorption time-of-flight mass spectrometry. Anal Chem, 1995, 67: 4557–4564

20 Noga M J, Asperger A, Silberring J. N-terminal H3/D3-acetylation for improved high-throughput peptide sequencing by matrix-assisted laser desorption/ionization mass spectrometry with a time-of-flight/time-of-flight analyzer. Rapid Commun Mass Spectrom, 2006, 20: 1823–1827

21 Zappacosta F, Annan R S. N-terminal isotope tagging strategy for quantitative proteomics: results-driven analysis of protein abundance changes. Anal Chem, 2004, 76: 6618–6627

22 Burt A D. C. L. Oakley Lecture. Cellular and molecular aspects of hepatic fibrosis. J Pathol, 1993, 170: 105–114

23 Azuma T, Koths K, Flanagan L, *et al*. Gelsolin in complex with phosphatidylinositol 4,5-bisphosphate inhibits caspase-3 and -9 to retard apoptotic progression. J Biol Chem, 2000, 275: 3761–3766

24 Neubauer K, Baruch Y, Lindhorst A, *et al*. Gelsolin gene expression is upregulated in damaged rat and human livers within non-parenchymal cells and not in hepatocytes. Histochem Cell Biol, 2003, 120: 265–275

25 Suhler E, Lin W, Yin H L, *et al*. Decreased plasma gelsolin concentrations in acute liver failure, myocardial infarction, septic shock, and myonecrosis. Crit Care Med, 1997, 25: 594–598

26 Attallah A M, Zahran F, Ismail H, *et al*. Immunochemical identification and detection of serum fibronectin in liver fibrosis patients with chronic hepatitis C. J Immunoassay Immunochem, 2007, 28: 331–342

27 Grieco A, Matera A, Di Rocco P, *et al*. Plasma levels of fibronectin in patients with chronic viral and alcoholic liver disease. Hepatogastroenterology, 1998, 45: 1731–1736

28 Perez Blanco F J, Rodriguez Cuartero A, Nunez Carril J, *et al*. Haptoglobin and hemopexin in the chronic hepatopathies. Rev Esp Enferm Apar Dig, 1982, 62: 20–24

29 Poli V, Silengo L, Altruda F, *et al*. The analysis of the human hemopexin promoter defines a new class of liver-specific genes. Nucleic Acids Res, 1989, 17: 9351–9365

30 Ratra R, Kar-Roy A, Lal S K. The ORF3 protein of hepatitis E virus interacts with hemopexin by means of its 26 amino acid N-terminal hydrophobic domain II. Biochemistry, 2008, 47: 1957–1969

31 Koukoulis G K, Shen J, Virtanen I, *et al*. Vitronectin in the cirrhotic liver: an immunomarker of mature fibrosis. Hum Pathol, 2001, 32: 1356–1362

32 Yamada S, Kobayashi J, Murawaki Y, *et al*. Collagen-binding activity of plasma vitronectin in chronic liver disease. Clin Chim Acta, 1996, 252: 95–103

33 Fan C, Stendahl U, Stjernberg N, *et al*. Association between orosomucoid types and cancer. Oncology, 1995, 52: 498–500

34 Sorensson J, Matejka G L, Ohlson M, *et al*. Human endothelial cells produce orosomucoid, an important component of the capillary barrier. Am J Physiol, 1999, 276: H530–534

35 Lovat L B, Persey M R, Madhoo S, *et al*. The liver in systemic amyloidosis: insights from 123I serum amyloid P component scintigraphy in 484 patients. Gut, 1998, 42: 727–734

36 Levo Y, Shalit M, Tur-Kaspa R. Serum amyloid P-component as a marker of liver disease. Am J Gastroenterol, 1982, 77: 427–430

37 Munthe-Fog L, Hummelshoj T, Honore C, *et al*. Immunodeficiency associated with FCN3 mutation and ficolin-3 deficiency. N Engl J Med, 2009, 360: 2637–2644

38 Le Y, Lee S H, Kon O L, *et al*. Human L-ficolin: plasma levels, sugar specificity, and assignment of its lectin activity to the fibrinogen-like (FBG) domain. FEBS Lett, 1998, 425: 367–370

39 Enjyoji K, Kato H. Purification and characterization of two isoforms of T-kininogens from rat liver microsomes. J Biochem, 1992, 111: 670–675

40 Giannelli G, Antonaci S. Immunological and molecular aspects of liver fibrosis in chronic hepatitis C virus infection. Histol Histopathol, 2005, 20: 939–944

41 Mehta A S, Long R E, Comunale M A, *et al*. Increased levels of galactose-deficient anti-Gal immunoglobulin G in the sera of hepatitis C virus-infected individuals with fibrosis and cirrhosis. J Virol, 2008, 82: 1259–1270

42 Ratziu V, Massard J, Charlotte F, *et al*. Diagnostic value of biochemical markers (FibroTest-FibroSURE) for the prediction of liver fibrosis in patients with non-alcoholic fatty liver disease. BMC Gastroenterol, 2006, 6: 6

43 Gobel T, Vorderwulbecke S, Hauck K, *et al*. New multi protein patterns differentiate liver fibrosis stages and hepatocellular carcinoma in chronic hepatitis C serum samples. World J Gastroenterol, 2006, 12: 7604–7612

44 West R B, Harvell J, Linn S C, *et al*. Apo D in soft tissue tumors: a novel marker for dermatofibrosarcoma protuberans. Am J Surg Pathol, 2004, 28: 1063–1069

45 Otogawa K, Kinoshita K, Fujii H, *et al*. Erythrophagocytosis by liver macrophages (Kupffer cells) promotes oxidative stress, inflammation, and fibrosis in a rabbit model of steatohepatitis: implications for the pathogenesis of human nonalcoholic steatohepatitis. Am J Pathol, 2007, 170: 967–980

46 Poon T C, Hui A Y, Chan H L, *et al*. Prediction of liver fibrosis and cirrhosis in chronic hepatitis B infection by serum proteomic fingerprinting: a pilot study. Clin Chem, 2005, 51: 328–335

47 Arteel G E. "HOPE" for the liver? Mechanistic insight into the role of the renin-angiotensin system in hepatic fibrosis. Hepatology, 2004, 40: 263–265

48 Xiao F, Wei H, Song S, *et al*. Polymorphisms in the promoter region of the angiotensinogen gene are associated with liver cirrhosis in patients with chronic hepatitis B. J Gastroenterol Hepatol, 2006, 21: 1488–1491

49 Schiavon L L, Schiavon J L, Filho R J, *et al*. Simple blood tests as noninvasive markers of liver fibrosis in hemodialysis patients with chronic hepatitis C virus infection. Hepatology, 2007, 46: 307–314

50 Mohamadnejad M, Montazeri G, Fazlollahi A, *et al*. Noninvasive markers of liver fibrosis and inflammation in chronic hepatitis B-virus related liver disease. Am J Gastroenterol, 2006, 101: 2537–2545

51 Lebensztejn D M, Skiba E, Sobaniec-Lotowska M, *et al*. A simple noninvasive index (APRI) predicts advanced liver fibrosis in children with chronic hepatitis B. Hepatology, 2005, 41: 1434–1435

52 Murata C, Watanabe T, Furuya H, *et al*. Aldehyde dehydrogenase 2 and β3-adrenergic receptor gene polymorphisms: their association with elevated liver enzymes and metabolic syndrome. Metabolism, 2003, 52: 1096–1101

53 Enomoto N, Takase S, Takada N, *et al*. Alcoholic liver disease in heterozygotes of mutant and normal aldehyde dehydrogenase-2 genes. Hepatology, 1991, 13: 1071–1075

54 Ferre N, Marsillach J, Camps J, *et al*. Genetic association of paraoxonase-1 polymorphisms and chronic hepatitis C virus infection. Clin Chim Acta, 2005, 361: 206–210

55 Shirai R, Hirano F, Ohkura N, *et al*. Up-regulation of the expression of leucine-rich α(2)-glycoprotein in hepatocytes by the mediators of acute-phase response. Biochem Biophys Res Commun, 2009, 382: 776–779

56 Heo S H, Lee S J, Ryoo H M, *et al*. Identification of putative serum glycoprotein biomarkers for human lung adenocarcinoma by multilectin affinity chromatography and LC-MS/MS. Proteomics, 2007, 7: 4292–4302

57 de Sa S V, Correa-Giannella M L, Machado M C, *et al*. Serpin peptidase inhibitor clade A member 1 as a potential marker for malignancy

in insulinomas. Clin Cancer Res, 2007, 13: 5322–5330

58 Sathe S, Sakata M, Beaton A R, *et al*. Identification, origins and the diurnal role of the principal serine protease inhibitors in human tear fluid. Curr Eye Res, 1998, 17: 348–362

59 Jiang H Y, Wang S X, Li X H, *et al*. The proteomics research on relational expressed serum proteins among the recovered SARS patients complicating avascular necrosis of femoral head (in Chinese). Zhonghua Yu Fang Yi Xue Za Zhi, 2008, 42: 522–526

60 Zhou M, Shen D, Head J E, *et al*. Ocular clusterin expression in von Hippel-Lindau disease. Mol Vis, 2007, 13: 2129–2136

61 Rodriguez-Pineiro A M, de la Cadena M P, Lopez-Saco A, *et al*. Differential expression of serum clusterin isoforms in colorectal cancer. Mol Cell Proteomics, 2006, 5: 1647–1657

62 Chen X, Halberg R B, Ehrhardt W M, *et al*. Clusterin as a biomarker in murine and human intestinal neoplasia. Proc Natl Acad Sci USA, 2003, 100: 9530–9535

63 Kang Y K, Hong S W, Lee H, *et al*. Overexpression of clusterin in human hepatocellular carcinoma. Hum Pathol, 2004, 35: 1340–1346

64 Meistermann H, Norris J L, Aerni H R, *et al*. Biomarker discovery by imaging mass spectrometry: transthyretin is a biomarker for gentamicin-induced nephrotoxicity in rat. Mol Cell Proteomics, 2006, 5: 1876–1886

65 Biroccio A, Del Boccio P, Panella M, *et al*. Differential post-translational modifications of transthyretin in Alzheimer's disease: a study of the cerebral spinal fluid. Proteomics, 2006, 6: 2305–2313

66 Barcelo-Batllori S, Kalko S G, Esteban Y, *et al*. Integration of DIGE

and bioinformatics analyses reveals a role of the antiobesity agent tungstate in redox and energy homeostasis pathways in brown adipose tissue. Mol Cell Proteomics, 2008, 7: 378–393

67 Saile B, Ramadori G. Inflammation, damage repair and liver fibrosis—role of cytokines and different cell types. Z Gastroenterol, 2007, 45: 77–86

68 Kershenobich Stalnikowitz D, Weissbrod A B. Liver fibrosis and inflammation. A review. Ann Hepatol, 2003, 2: 159–163

69 Yip T T, Cho W C, Cheng W W, *et al*. Application of ProteinChip array profiling in serum biomarker discovery for patients suffering from severe acute respiratory syndrome. Methods Mol Biol, 2007, 382: 313–331

70 Spisak S, Tulassay Z, Molnar B, *et al*. Protein microchips in biomedicine and biomarker discovery. Electrophoresis, 2007, 28: 4261–4273

71 Zhang G, Terry A V Jr, Bartlett M G. Liquid chromatography/tandem mass spectrometry method for the simultaneous determination of olanzapine, risperidone, 9-hydroxyrisperidone, clozapine, haloperidol and ziprasidone in rat plasma. Rapid Commun Mass Spectrom, 2007, 21: 920–928

72 Kim K, Kim S J, Yu H G, *et al*. Verification of biomarkers for diabetic retinopathy by multiple reaction monitoring. J Proteome Res, 2010, 9: 689–699

73 Boersema P J, Raijmakers R, Lemeer S, *et al*. Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics. Nat Protoc, 2009, 4: 484–494