Novel Serum Biomarker Candidates for Liver Fibrosis in Hepatitis C Patients

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**Background:** Liver biopsy is currently the gold standard for assessing liver fibrosis, and no reliable noninvasive diagnostic approach is available. Therefore a suitable serologic biomarker of liver fibrosis is urgently needed.

**Methods:** We used a proteomics method based on 2-dimensional gel electrophoresis to identify potential fibrosis biomarkers. Serum samples from patients with varying degrees of hepatic scarring induced by infection with the hepatitis C virus (HCV) were analyzed and compared with serum from healthy controls.

**Results:** We observed the most prominent differences when we compared serum samples from cirrhotic patients with healthy control serum. Inter-α-trypsin inhibitor heavy chain H4 (ITIH4) fragments, α1 antichymotrypsin, apolipoprotein L1 (Apo L1), prealbumin, albumin, paraoxonase/arylesterase 1, and zinc-α2-glycoprotein were decreased in cirrhotic serum, whereas CD5 antigen-like protein (CD5L) and β2 glycoprotein I (β2GPI) were increased. In general, α2 macroglobulin (α2M) and immunoglobulin components increased with hepatic fibrosis, whereas haptoglobin and complement components (C3, C4, and factor H-related protein 1) decreased. Novel proteins associated with HCV-induced fibrosis included ITIH4 fragments, complement factor H-related protein 1, CD5L, Apo L1, β2GPI, and thioester-cleaved products of α2M.

**Conclusions:** Assessment of hepatic scarring may be performed with a combination of these novel fibrosis biomarkers, thus eliminating the need for liver biopsy. Further evaluation of these candidate markers needs to be performed in larger patient populations. Diagnosis of fibrosis during early stages will allow early treatment, thereby preventing fibrosis progression.

Chronic hepatic disease damages the liver, and the resulting wound-healing process can lead to liver fibrosis and the subsequent development of cirrhosis. One of the leading causes of hepatic fibrosis and cirrhosis is infection with the hepatitis C virus (HCV)². HCV is a major human pathogen, infecting more than 170 million individuals, approximately 3% of the world’s population (1). Approximately 70% of those infected become chronic carriers and are at severe risk of developing liver fibrosis and cirrhosis. Of the patients with HCV-induced cirrhosis, 2%–5% develop hepatocellular carcinoma (HCC) (1).

Hepatic fibrosis is characterized by the excessive buildup of scar tissue in the liver. This accumulation in extracellular matrix (ECM) changes hepatic morphology. Collagen is the primary component of the ECM in fibrosis. Other ECM components, including laminin, hyaluronan, elastin, and fibronectin, also increase (2). As fibrotic liver diseases advance, cirrhosis occurs and the liver becomes nodular. Expression of collagens can increase up to 10-fold in cirrhosis (3).

Kupffer cells (liver macrophages) in the blood pass through the hepatic sinusoids and in fibrosis become activated by adverse stimuli such as viruses or toxins. Cytokines such as transforming growth factor β1 (TGF-β1) are released from the Kupffer cells, which activate the cells responsible for ECM production. These hepatic stellate cells (HSCs) also produce TGF-β1 and other cytokines as well as angiotensin II. These profibrogenic factors, along with hepatocyte growth factor (HGF) in the ECM, further activate HSCs in hepatic scarring (2–4).

Excess ECM can be removed by HSC-synthesized matrix metalloproteinases, which are activated by the

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2 Nonstandard abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; ECM, extracellular matrix; TGF-β1, transforming growth factor β1; HSC, hepatic stellate cell; HGF, hepatocyte growth factor; 2D, 2 dimensional; MS, mass spectroscopy; MS/MS, tandem MS; ITIH4, inter-α-trypsin inhibitor heavy chain H4; Apo L1, apolipoprotein L1; CD5L, CD5 antigen-like protein; β2GPI, β2 glycoprotein I; α2M, α2 macroglobulin.
serine protease plasmin. However, repeated injury to the liver, such as from toxins or hepatic viruses, leads to fibrogenesis prevailing over fibrolysis. As a result, ECM production increases and the secretion and activity of matrix metalloproteinases decreases. In fibrosis, activated HSCs synthesize and release tissue inhibitors of metalloproteinases. TGF-β1 stimulates these inhibitors and decreases plasmin concentrations (2, 3).

Liver biopsy followed by histologic examination is the primary tool for the diagnosis and assessment of fibrosis, but this method has a number of well-documented limitations and disadvantages (2). The invasive procedure is expensive and leads to severe pain in up to 40% of patients; in rare cases death can occur. In addition, in cases in which hepatic scarring is not homogenous throughout the liver, the rate of false negatives from liver biopsy is 15%–20%, with sampling error occurring most frequently when small biopsies (<10 mm) are analyzed (2, 5).

Noninvasive diagnostic methods have been proposed as alternatives or adjuncts to biopsies. These methods include measurement of glycoproteins and glycans and assessment of increased hepatic elasticity. Examples of secreted serum biomarkers include the N-terminal propeptide of type III collagen, hyaluronic acid, tissue inhibitor of metalloproteinase type 1, and YKL-40 (2). Overlap between the intermediate stages of fibrosis may occur with these noninvasive methods, and results for fibrosis patients can be similar to those for healthy individuals. Currently the most reliable serologic assessment of liver fibrosis uses a panel of 5 markers: a2 macroglobulin (a2M), haptoglobin, apolipoprotein A1, γ-glutamyl transpeptidase, and bilirubin (4). However, this test eliminates the need for biopsy in only 26% of patients and does not accurately predict the presence or absence of fibrosis (6).

A reliable, noninvasive method for serologic assessment of hepatic fibrosis is urgently needed. Ideally the biomarker(s) should enable diagnosis of fibrosis in the early stages of hepatic fibrosis, allowing therapeutic intervention to prevent progression to cirrhosis and HCC. Here we describe a study that used gel-based proteomic techniques to identify biomarkers for hepatic fibrosis by analyzing serum from patients with varying stages of HCV-induced hepatic fibrosis and cirrhosis and from healthy individuals. We also investigated possible relationships between potential novel biomarkers and viral-mediated hepatic fibrosis.

Materials and Methods

SAMPLES FOR ANALYSIS
Serum samples were obtained from healthy control individuals and from HCV-infected patients who were not undergoing treatment. The study was approved by the Central Oxford Research Ethics Committee (No. 98.137). The degree of hepatic fibrosis in each HCV-infected patient was determined using the Ishak scoring scale (7).

Patients in each of the following categories were analyzed (the numbers in brackets indicate the Ishak fibrosis score): healthy controls [0], mild fibrosis [1], moderate fibrosis [3], and cirrhosis [6]. Four individuals were analyzed for each fibrosis stage, with the exception of moderate fibrosis, for which serum samples were available from only 3 patients.

2-DIMENSIONAL PAGE
Serum samples were diluted in 5 mol/L urea, 2 mol/L thiourea, 4% (wt/vol) CHAPS, 65 mmol/L dithiothreitol, 2 mmol/L tributylphosphine, 150 mmol/L nondetergent sulfobetaine-256 (NDSB-256), and 0.0012% (wt/vol) bromphenol blue. The protein solution was mixed with 0.45% (by volume) pH 2–4 ampholytes, 0.45% (by volume) pH 9–11 ampholytes, and 0.9% (by volume) pH 3–10 ampholytes (SERVALYT®, SERVA). Samples were spun at 16 000g for 15 min; 375 μL of supernatant (containing 500 μg serum) was used for 2-dimensional (2D)-PAGE with 18-cm pH 3–10 nonlinear immobilized pH gradient DryStrips (GE Healthcare). Rehydration was performed for 20 h, followed by isoelectric focusing with a Multiphor (GE Healthcare) at 300 V for 2 h and then at 3500 V up to 75 kVh. After isoelectric focusing, immobilized pH gradient strips were incubated in equilibration solution 4 mol/L urea, 2 mol/L thiourea, 50 mmol/L Tris-HCl (pH 6.8), 30% (by volume) glycerol, 2% (wt/vol) SDS, 130 mmol/L dithiothreitol, 0.002% (wt/vol) bromphenol blue) for 15 min. Proteins were then separated by 9%–16% (wt/vol) SDS-PAGE gradient gels using 20 mA per gel for 1 h, followed by 40 mA per gel for 4 h with the temperature maintained at 10 °C. Gels were stained with the fluorescent dye OGT 1238 (Oxford Glycosciences) as previously described (8), and 16-bit images with a resolution of 200 μm were captured on an Apollo II laser scanner (Oxford Glycosciences).

DIFFERENTIAL IMAGE ANALYSIS
Gel images were analyzed using a custom version of the Melanie II software from Oxford Glycosciences as previously described (8). For differential analysis, samples from healthy controls were compared with those from patients with each of the 3 stages of hepatic scarring. After spot detection, artifacts were removed and features were manually edited only if spot splitting was required. Accurate spot matching was used to create 3 synthetic images for analyses between the control gels and each of the 3 stages of hepatic scarring. These synthetic images contained all of the features in the differential analyses. The absorbance of each feature was determined by summing pixels within the feature boundary, and the volume was determined by integrating this absorbance over the area of the feature. All statistical calculations were based on the percentage volume of the features. Changes in protein expression were determined as a ratio of the mean percentages of feature volumes. Only features present in at least 66% of individual gels belonging to either the
control or hepatic-scarring groups were considered for differential analysis (i.e., present in at least 2 of the 3 moderate fibrosis gels and 3 of the 4 control, mild fibrosis, and cirrhosis gels). Differential expression of a protein present in both the control and hepatic-scarring gels was considered significant when the fold change was at least 2 and \( P \) was no more than 0.05 (with 95% confidence) after the application of a rank-sum test on percentage spot volumes as previously described \( (8) \). In addition, all features displayed as differentially expressed by the software were further validated by visualizing the features across all gels in a montage format.

**Mass Spectrometric Analysis**
Excised protein features were digested (DigestPro, Intavis), and the tryptic peptides were analyzed using a Q-TOF 1 mass spectrometer (Micromass) coupled to a CapLC (Waters). Peptides were concentrated and desalted on a 300-\( \mu \)m inner diameter/5-mm C18 precolumn and resolved on a 75-\( \mu \)m inner diameter/25-cm C18 PepMap analytical column (LC packings) with a 45-min 5%-95% acetonitrile gradient containing 0.1% formic acid at a flow rate of 200 nL/min. Spectra were acquired in positive mode, and the mass spectrometry (MS) to tandem MS (MS/MS) switching was controlled in an automatic data-dependent fashion, with a 1-s survey scan followed by 3 1-s MS/MS scans. Ions selected for MS/MS were excluded from further fragmentation for 2 min. Spectra were processed using Proteinlynx Global server 2.1.5 and searched against the SWISS-PROT database (release 20040206) using MASCOT (Matrix Science) restricted to the human taxonomy and allowing carbamidomethyl cysteine as a fixed modification and oxidized methionine as a variable modification. Data were searched allowing 0.5-Da error to accommodate calibration drift and up to 2 missed tryptic cleavage sites. All data were checked for consistent error distribution and all positive identifications were checked manually.

**Results**
A synthetic gel image representative of all the features in the differential analysis comparing samples from controls and all cirrhosis patients is shown in Fig. 1. Original gel images for all samples are shown in Fig. 1 of the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol53/issue10 Synthetic gel images for the comparison of control samples to those from patients with mild and moderate fibrosis are shown in Supplemental Data Fig. 2. Zoomed images of the gel regions depicting differential changes for some of the potential novel fibrosis biomarkers are shown in Fig. 2. A total of 53 statistically significant differentially expressed features were excised, digested with trypsin, analyzed by LC-MS/MS, and identified using the SwissProt database. A total of 83 differentially expressed proteins were identified and are listed in Table 1 of the online Data Supplement. Table 1 shows a summary of selected differentially expressed proteins that have been classified according to their function. In cirrhotic serum we observed decreased expression of inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) fragments, \( \alpha 1 \) antichymotrypsin, apolipoprotein L1 (Apo L1), prealbumin, albumin, paraoxonase/arylesterase 1, and zinc-alpha2-glycoprotein and increased expression of CD5 antigen-like protein (CD5L) and \( \beta 2 \) glycoprotein 1 (\( \beta 2 \)GPI). In general, a2M and immunoglobulin components increased with hepatic scarring, whereas haptoglobin and complement components decreased. Further information about the proteins, including amino acid sequence and sites of glycosylation, was derived from the ExPASy database (http://www.expasy.ch/).

The peptide sequences identified by MS for ITIH4, a2M, and complement C3 are shown in Fig. 3 of the online Data Supplement. Spot features for ITIH4 were greater in control samples than in serum from patients with cirrhosis. This liver-synthesized glycoprotein was differentially expressed in 3 areas of the gels: 2 spots at approximately 35 kDa were absent in the cirrhotic gels but present in healthy controls; 1 smear at approximately 84 kDa showed decreased expression in cirrhotic serum samples compared with healthy controls (Fig. 2B). This inhibitor is cleaved by kallikrein into 35- and 70-kDa fragments \( (9) \). Peptides identified by MS for the spots at 35 kDa and the smear at 84 kDa relate to the 35-kDa and 70-kDa fragments, respectively (see Fig. 3A in the online Data Supplement).

Serum concentrations of a2M were found to increase with hepatic fibrosis development (Fig. 2C). a2M was found to change in 3 areas of the gel, and peptide information confirmed that these corresponded to uncleaved a2M, the N-terminal end of a2M preceding a thioester site, and the C-terminal end of a2M after a thioester site (see Fig. 3B in the online Data Supplement). Compared to healthy control serum, the thioester-cleaved C-terminal end of a2M was found to change by more than 2-fold in percentage spot volume only in cirrhotic serum. The thioester-cleaved N-terminal end of a2M changed by more than 2-fold mostly in cirrhotic serum and with fewer glycoforms in serum from moderate fibrosis patients. Uncleaved a2M was differentially expressed in all stages of fibrosis (see Table 1 in online Data Supplement).

Fragments for complement C3 and C4 were decreased in the serum samples of patients with moderate fibrosis and cirrhosis compared with healthy controls. In the case of complement C3, a fragment observed at 49 kDa, pl 6.9 contained peptides corresponding to the \( \alpha \)-chain of C3 preceding a thioester site (see Fig. 3C in the online Data Supplement).

**Discussion**
Liver biopsy is currently the gold standard for diagnosing hepatic fibrosis, but a reliable and less invasive serologic diagnostic tool is needed. We used 2D-PAGE–based proteomics to identify serum biomarkers for HCV-in-
duced liver fibrosis. A representation of the possible interrelationship between these proteins and the already established pathways for ECM remodeling is shown in Fig. 4 in the online Data Supplement. Although multivariate statistical analyses of these interrelated proteins would allow linkage of the expression of the identified candidate biomarkers to changes in expression of other proteins, such speculation is beyond the purpose of this study, which focused on the identification of novel serum biomarkers.

The potential involvement of each of these proteins with HCV-induced hepatic scarring is briefly discussed here and in more detail in Fig. 4 of the online data supplement.

CD5L belongs to the same family as CD5 and is associated with IgM (11–13). HCV viral load positively correlates with CD5/H11001 B-cell expansion (14). The observed increase in serum CD5L in cirrhotic patients may be associated with HCV infection rather than cirrhosis.

For some inter-α-trypsin inhibitor proteins, the heavy chain stabilizes the ECM (15). ITIs can inhibit and increase the plasma clearance of enzymes involved in fibrolysis (16, 17). To our knowledge, ITIH4 has never been described in hepatic cirrhosis. Kallikrein can cleave ITIH4 into 35- and 70-kDa fragments (9), which were observed in healthy controls (see Fig. 3A in the online Data Supplement), suggesting that kallikrein is decreased in cirrhosis, a finding that is consistent with previous studies (18).

α2M cleaves at its thioester site when binding to fibrolytic enzymes (19). Our results indicate that this thioester cleavage may increase with fibrosis development (Fig. 2C, and see Fig. 3B and Table 1 in the online Data Supplement). Although increased α2M is already established as an indicator of hepatic scarring (4), our
Fig. 2. Magnified regions of the gels showing changes for some of the potential novel fibrosis biomarkers.

(A), CD5L [O43866] increases in serum from cirrhotic patients. (B), apparent absence of cleaved interα-trypsin inhibitor heavy chain H4 [Q14624] in serum from cirrhosis patients. The spots for the 70 and 35-kDa fragments are highlighted in the upper and lower panels respectively. (C), thioester cleavage of α2 macroglobulin [P01023]. U, uncleaved α2M; H, high-molecular-weight fragment from thioester cleavage; L low-molecular-weight fragment from thioester cleavage. (D), β2GPI [P02749] increases in cirrhosis. (E), apparent absence of Apo L1 [O14791] in cirrhosis.
results suggest that additional analysis of the thioester cleavage products may give a more reliable indication of hepatic fibrosis activity.

HCV may cause disorders in lipid metabolism leading to steatosis and possibly fibrosis (3). Proteins associated with lipid metabolism were found to be differentially expressed in hepatic scarring.

β2GPI is associated with chylomicron remnants, which show decreased uptake into hepatocytes in fibrosis (20, 21). This decreased uptake may produce an increase of β2GPI-bound chylomicron remnants in the circulation, consistent with the observed β2GPI increase. To our knowledge, this hypothesis is the 1st to suggest that increased concentrations of β2GPI may be related to fibrosis.

The function of Apo L1 is unknown, and Apo L1 has never been reported in relation to hepatic disease. Apo L1 positively correlates with hypertriglyceridemia and hypercholesterolemia (22). Hypocholesterolemia has been described in viral-mediated cirrhosis (23), a finding consistent with the apparent absence of Apo L1 in cirrhotic patients.

Decreased paraoxonase/arylesterase 1 has been observed in cirrhosis and HCC (24). Its activity decreases in chemically induced cirrhosis in rats and is inversely associated with lipid peroxidation (25); however, decreased paraoxonase/arylesterase 1 is not specific to liver scarring (10). Zinc-α2-glycoprotein can bind to the F protein encoded by HCV (26) and increases fat losses in some cancers (27), although no association of zinc-α2-glycoprotein with HCC has been reported.

The decrease in complement C3 and C4 fragments has already been described in cirrhosis (28). C3 also decreases in HBV-induced hepatitis and HCC (29). Plasmin cleaves the α-chain of C3 at a thioester site; this cleavage results in a fragment of approximately 39 kDa and pI 7, characteristics consistent with the protein spot we observed, considering that this fragment has 1 potential N-glycosylation site and all the peptides identified span this fragment (see Fig. 3C in the online Data Supplement Fig. 3C). The decreased expression of this fragment in fibrosis may indicate less plasmin-mediated cleavage of the complement C3 α-chain, a finding consistent with hepatic scarring. The decrease in complement factor H–related protein 1 may be related to lipid metabolism disruption (30).

Our finding of decreased α1 antichymotrypsin is consistent with previous reports (31). Chymotrypsin can convert angiotensin I to angiotensin II (32). Decreased α1 antichymotrypsin suggests increased angiotensin II, which activates HSCs, resulting in ECM synthesis (2).
The observed decrease in haptoglobin, prealbumin, and albumin in hepatic scarring and increase in IgG heavy constant chain, Ig k/λ chain regions, and IgA heavy chain is consistent with previous studies (4, 33–35). Our finding of increased β2 microglobulin in cirrhosis is also consistent with previous reports but is unreliable because increased β2 microglobulin is more closely correlated to renal damage (36).

Some of the proteins we identified share similar functions and can be grouped into categories related to hepatic scarring (Table 1). A few of the changes identified are not specific to fibrosis and may have other causes. For example, a2M and haptoglobin change in sickle cell anemia (37), hepatotoxic drugs decrease complement (38), and CD5L may correlate with viral infection rather than hepatic scarring (14). Prealbumin is unlikely to be suitable as a fibrosis biomarker because dietary factors can also affect this protein (34). However, some of the proteins identified from the differential analysis, or preferably a combination of proteins, may be useful in assessing hepatic fibrosis. Of the novel biomarkers, the proteins that appear to be most closely related to liver fibrosis are Apo L1, β2GPI, the thioester cleaved products of a2M, and the fragments of ITIH4. With the exception of β2GPI changing in HCC (39), these candidates have not been reported to display similar expression changes in other disease states.

Because of the time-consuming nature of thorough image analysis combined with sample reproducibility, we chose to analyze gels of different individuals rather than a subset of replicates. The serum proteome tends to differ even among patients with the same extent of hepatic scarring, largely because of variations in diet and fasting or postprandial states (40). Running and analyzing a subset of replicates may enhance such variables, which are unrelated to liver pathology. Biological reproducibility was also a limiting factor because of the difficulty in obtaining multiple HCV-induced fibrosis samples from patients who were not on any form of HCV therapy and the inability to keep certain factors constant. Therefore in addition to dietary variables, patients were not sex or age matched. Our results were not compromised by changes in these variables, however, because these variables also would not be controlled if these potential biomarkers were used to monitor fibrosis scores in patients. A fibrosis biomarker should ideally be useful in testing a wide range of patients. The analysis of several serum samples for each fibrosis stage helps to reduce variances caused by these uncontrolled variables.

Although many of the changes identified were observed only when we compared controls with cirrhotic patients, these changes may also be present in the earlier stages of fibrosis but in amounts below the detection limits of the fluorescent stain used. The dependability of these proteins as biomarkers for fibrosis is currently being validated by Western blotting and ELISAs with a greater number of serum samples. The use of these techniques to assess each of the candidate biomarkers may reveal a dependable biomarker that changes during the course of hepatic scarring. Thus these validation studies may reveal a protein, or combination of proteins, that can be used to reliably assess all stages of liver scarring. Ultimately such biomarkers would aid clinicians in diagnosing fibrosis during the early stages, eliminating the need for liver biopsy and allowing early treatment, thereby preventing fibrosis progression. In addition, a diagnostic assay could be used for longitudinal studies to monitor fibrosis development in individuals or fibrosis reduction during therapy.

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