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Identification and development of fucosylated glycoproteins as biomarkers of primary hepatocellular carcinoma

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Abstract

Changes in N-linked glycosylation are known to occur during the development of cancer. For example, we have previously reported changes in N-linked glycosylation that occur with the development of hepatocellular carcinoma (HCC) and, through the use of glycoproteomics, identified many of those proteins containing altered glycan structures. To advance these studies and further explore the glycoproteome, we performed N-linked glycan analysis from serum samples depleted of the major acute phase proteins, followed by targeted lectin extraction of those proteins containing changes in glycosylation. Using this method, changes in glycosylation, specifically increased amounts of core and outer arm fucosylation were observed in the depleted samples. The identities of those proteins containing core and outer arm fucose were identified in the serum patients with HCC. The usefulness of some of these proteins in the diagnosis of HCC was determined through the analysis of over 300 patient samples using a high-throughput plate based approach. Greatest performance was achieved with fucosylated hemopexin, which had an AUROC of 0.9515 with an optimal sensitivity of 92% and a specificity of 92%.

INTRODUCTION

Infection with hepatitis B virus (HBV) (and/or hepatitis C virus) is the major etiology of hepatocellular cancer (HCC) ^{1–3}. Both HBV and HCV cause acute and chronic liver infections and most chronically infected individuals remain asymptomatic for many years. Nearly 25% of all chronic carriers eventually develop untreatable liver cancer, and it is estimated that over one million people worldwide die because of HBV/HCV associated liver cancer⁴. Indeed, HBV and HCV infections are associated with over 80% of all HCC cases worldwide and can be as high as 96% in regions where HBV is endemic⁵.

The chronic infection of HBV or HCV is asymptomatic and monitored by regular (usually annual or biannual) physical assessments, serum liver function tests (LFTs) and ultrasound

imaging for detection of small masses in the liver (Hepatitis B foundation, 1994). The ultrasound imaging is very expensive, making its routine use prohibitive. Moreover, ultrasounds effectiveness is limited, since the appearance of masses at least 3 cm in size is required for detection, and this often occurs at a stage when the prognosis is very poor^{6, 7}. The correlation between elevated serum concentrations of alpha feto protein (AFP) and the occurrence of HCC has provided a useful surrogate marker for disease⁸. Levels of AFP exceeding 50ng/ml occur in 40–60% of the cases of HCC at the time of diagnosis⁹. However, AFP levels may actually fluctuate wildly in chronically infected individuals and are influenced by a number of non-malignant physiological events⁹. Early surgical and chemotherapeutic intervention is an afflicted individual's best hope^{10–12}. Unfortunately, it is impossible to detect HCC early by current methods. Thus, there is a clear and urgent need for non-invasive, reliable methods of detecting HCC as early as possible.

Protein glycosylation is one of the most common modifications made to proteins and occurs both co- and post-translationally¹³. Sugars (glycans) can be attached to proteins either via an amide group (N-linked glycosylation) or a hydroxyl group (O-linked glycosylation). N- and O- linked glycosylation are distinct protein modifications and have different biosynthetic pathways, and possibly independent functions¹⁴.

Cellular factors play a major role in controlling the type of glycosylation reactions that can occur. Consequently the physiological state of the cell may affect the glycosidase and transferase levels within a particular cell. Indeed, changes in the cell cycle affect the levels of glycan transferases and subsequent structure of the glycan chains¹⁵. Since glycan processing is sensitive to the cellular environment, alterations in glycosylation may be indications of changes within the cell. For example, the glycosylation of AFP changes in various disease states, including cirrhosis of the liver and liver cancer^{9, 16, 17}. Specifically, AFP becomes fucosylated and this alteration is the basis of the diagnostic test called AFP-L3¹⁸. This increase in fucosylation is not limited to AFP, and the literature is now full (by us and others) of examples of increased fucosylation and its association with liver cancer. However, the majority of this analysis has been performed *individually* on just a handful of the most abundant serum glycoproteins^{19–21}. As specific fucosylated glycoforms may be valuable biomarkers, it was our desire to identify all of these glycoproteins, as they may be useful as early diagnostic markers of cancer. Thus, in this report we have determined that changes in glycosylation associated with liver cancer can be detected via a quantitative and sensitive HPLC based approach and we have identified many of those glycoproteins whose fucosylation changes with liver cancer. The potential use of these markers in the detection of liver cancer is discussed.

Materials and methods

Human Subjects

Serum samples were obtained from Saint Louis University School of Medicine under a study protocol approved by the Saint Louis University Institutional Review Board and written informed consent was obtained from each subject. Demographic and clinical information was obtained, and a blood sample was collected from each subject in a serum separator tube, spun within 2 hours and serum stored at –80°C until testing. For the HCC

group, consecutive patients were enrolled from the Saint Louis University Liver Cancer Clinic using criteria for HCC diagnosis established for the HALT-C trial. Subjects either had HCC on biopsy, a new hepatic defect showing vascular enhancement on one imaging modality (ultrasound [US], magnetic resonance imaging [MRI], or computed tomography [CT]) with AFP > 1000 ng/ml or presumed HCC. Subjects were presumed to have HCC if they had a discrete hepatic defect on US with AFP < 1000 ng/ml and either 2 other scans (MRI, CT, angiography) indicating malignancy with at least 1 of the following characteristics: Hypervascularity, arterial to portal vein shunts, portal vein thrombosis near the defect, tumor in the portal vein or 1 other scan (MRI or CT) showing features characteristic of HCC and either an increase in size over time after initial discovery (at least doubling if less than 1 cm) or an increase in AFP to > 200 ng/ml. Tumor staging was determined using the United Network of Organ Sharing-modified TNM staging system for HCC. For the cirrhosis group, patients with Hepatitis C and biopsy proven cirrhosis were enrolled. All cirrhotic controls were screened for HCC using US, CT or MRI prior to enrollment.

Seppro and AAL serum fractionation

100 µl of sera was depleted of the albumin, IgG, alpha-1-antitrypsin, IgA IgM, transferrin, haptoglobin, alpha-1-acid glycoprotein, alpha-2-macroglobulin, Apolipoprotein AI& AII and fibrinogen using the GenWay Seppro Column and Reagent Kit (Genway San Diego, CA) according to the manufacturer's instructions. The Seppro unbound fractions were further fractionated using agarose-bound *Aleuria Aurantia* Lectin affinity chromatography (Vector Labs, Burlingame, CA) as we have done previously^{22, 23}.

Glycan analysis

10% acrylamide gel plugs are dehydrated in acetonitrile, rehydrated in 20mM ammonium bicarbonate and dehydrated once again in acetonitrile. The gel plugs are then dried in a speed vac. The Seppro depleted and Seppro bound proteins were concentrated and the equivalent of 5 µl worth of serum adsorbed into the dehydrated gel plugs, denatured with DTT at 100 degrees Celsius for five minutes, allowed to cool and alkylated in the dark for thirty minutes with iodoacetamide. The gel plugs were fixed in a solution of 30% ethanol 7% acetic acid for one hour. The gel plugs were washed by dehydration in acetonitrile, rehydration in 20mM ammonium bicarbonate and dehydration with acetonitrile, than dried in a speed-vac. PNGase F was diluted with 20mM ammonium bicarbonate pH 7 and allowed to adsorb into the gel plug. The gel plug was then covered with the same solution and allowed to incubate overnight at 37 degrees Celsius. The glycans were eluted from the gel plug by sonication in Milli-Q water three times and the elutant pooled, dried down and labeled with a 2AA dye (Ludger, Oxford, UK) according to manufactures instructions. The glycans were then cleaned up using paper chromatography and filtered using a 0.22 micron syringe filter. Fluorescently labeled glycans were subsequently analyzed by HPLC using a normal phase column (TSK amide 80 column). The mobile phase consisted of Solvent A (50mM ammonium formate, pH 4.4) and Solvent B (acetonitrile) and the gradient used was as follows: linear gradient from 20%–58% Solvent A at 0.4ml/minute for 152 minutes followed by a linear gradient from 58%–100% Solvent A for the next 3 minutes. The flow rate was increased to 1.0 ml/minute and the column washed in 100% Solvent A for 5

minutes. Following the wash step the column was equilibrated in 20% Solvent A for 22 minutes in preparation for the next sample run. HPLC analysis was performed using the Waters Alliance HPLC System, complemented with a Waters fluorescence detector, and quantified using the Millennium Chromatography Manager (Waters Corporation, Milford, MA). Glycan structures were identified by the calculation of GU value as described previously and through comparison to known standards and by sequential exoglycosidase digestion²⁴.

Mass spectrometry

LC MS/MS Analysis—Mass spectrometry was completed on an LTQ ion trap mass spectrometer (ThermoFisher, San Jose, CA) using the microspray source from ThermoFisher, spray voltage was 2KV. Separation was achieved using an Ultimate 3000 microflow HPLC (Dionex, Sunnyvale, CA). 5ul of sample was injected onto a C18 trap column (Dionex), and then transferred to a 75×150um C18 column (Dionex). The gradient was a 5–65% buffer A to B over 2 hours, where buffer A is 5% acetonitrile + 0.5% acetic acid and buffer B is 90% acetonitrile + 0.5% acetic acid at a flow rate of 250 nl/min. The LTQ was programmed to either perform MS/MS fragmentation on the top 5 ions. Data exclusion was enabled with a peak list of 100, for 360 seconds, width of .75Da, when ion was seen twice within 30 seconds.

Data analysis and interpretation—Mass spectrometry data (spectra) was searched using BioWorks 3.2 (ThermoFisher, San Jose, CA) using the CDS non-redundant database (Celera, Alameda, CA) along with Swissprot additions (expasy.org). Search criteria used were $XC > 2.0$ for $z=1$, $XC > 2.5$ for $z=2$, and $XC > 3$ for $z=3$, percent ions $> 50\%$, peptide probability $< 1e^{-3}$ using a tryptic digest restriction. Peptides that scored above the threshold value were manually verified.

Lectin FLISA

Monoclonal antibodies are fucosylated and are reactive with fucose binding lectins. Hence they must be modified prior to analysis via the Lectin-FLISA. Briefly, to remove the fucosylation of the capture antibody (Mouse anti-human fetuin-A or rabbit anti-human hemopexin, Bethyl Laboratories, Montgomery, TX), antibody was incubated with 10mM sodium periodate for 1 hour at 4°C. An equal volume of ethylene glycol was added and the oxidized antibody brought to a concentration of 10µg/mL with sodium carbonate buffer, pH 9.5. Antibody (5 µg/well) was added to the plate and following incubation washed with 0.1% Tween 20/PBS 7.4 and blocked overnight with 3% BSA/PBS. For analysis, 5 µl of serum was diluted in 95 µL of Heterophilic Blocking tubes (Scantibodies Laboratory, Inc. Santee, CA 92071 USA) and incubated at room temperature for 1 hour. Subsequently, samples were added to the plates for 2 hours and washed 5 times in lectin incubation buffer (10mM Tris pH 8.0, 0.15M NaCl, 0.1% Tween 20) before fucosylated protein was detected with a biotin conjugated *Aleuria aurantia* (AAL) lectin (Vector Laboratories, Burlingame, CA). Bound lectin was detected using IRDyeTM 800 Conjugated streptavidin and signal intensity measured using the Odyssey® Infrared Imaging System (LI-COR Biotechnology, Lincoln, Nebraska). In all cases sample intensity was compared to commercially purchased human serum (Sigma Inc., St Louis, MO.).

Results

Removal of highly abundant proteins: Depletion of major serum proteins from human serum

There are about 12 plasma proteins that make up about 95 percent of the protein mass of plasma (the same holds true for sera), the majority of which are glycoproteins. However, the other proteins in serum that may be important biomarkers for health and disease are present in much lower abundance. The difference in concentration can be greater than 10-fold, making detection of low-abundance proteins a considerable challenge. Thus by removing the most abundant proteins associated with serum, we may find markers that may be more specific towards the development of HCC. Indeed, many of the fucosylated proteins identified so far consist mainly of these abundant acute phase proteins. Therefore to ensure consideration of less abundant proteins, the most abundant proteins were removed prior to glycan analysis, lectin extraction and proteomic analysis. To remove the major serum proteins, we utilized the GenawaySeppro IGY-12 microbead system to specifically remove the most abundant proteins from serum. The Seppro™ IGY-12 bead system is comprised of 12 types of IgY polyclonal antibody microbeads. These antibody microbeads are optimized in a mixture that can simultaneously remove the top 12 most abundant proteins that make up about 95 percent of the protein mass of plasma: albumin (HSA), IgG, Fibrinogen, serotransferrin, IgA, IgM, Haptoglobin, α -2-Macroglobulin, α -1-Acid Glycoprotein, α -1-Antitrypsin and HDL (Apo A-I & Apo A-II). As figure 2 shows, we can successfully remove >98% of the albumin, alpha 1 anti-trypsin (AAT) and IgG from human serum using this system. Similar results were obtained with the other proteins extracted using the Seppro™ MIXED12 system. In addition, as figure 2B shows, while these proteins have been removed, no major loss of other proteins was observed. That is, the vast majority of AFP and Apo-D were still found in the Seppro™ unbound fraction (figure 2b). We have performed western blots for many proteins and have confirmed that the vast majority of protein is found where we expect it to be found. That is, proteins that are being extracted by the Seppro system are found predominantly bound to the beads and proteins that should not be extracted such as AFP, Apo-D, HBsAg, Gp-73, hemopexin, and ceruloplasmin are all found in the flow through (data not shown).

Glycan analysis of fractionated serum samples

Comparative N-linked glycan analysis was performed on the serum samples from the healthy, cirrhotic or HCC patients to identify those glycan structures that are altered with the development of HCC in the depleted sera. Briefly, pooled samples from healthy individuals, individuals with cirrhosis or individuals with cirrhosis plus HCC were depleted of 12 highly abundant serum proteins as described in figure 1. Following depletion of these proteins, glycan analysis was performed on both the abundant proteins (bound fraction) and on the less abundant proteins (unbound). The results are shown in figure 3. The main difference in glycosylation observed in patients with HCC was an increase in the level of a core fucosylated bi-antennary glycan (FcA2G2), which increased from 5.8% in the healthy patients to 8.5% in the pooled cirrhotic serum and up to 10% in patients with HCC. Increases were also observed in the level of a core fucosylated bi-antennary glycan with two sites of outer-arm fucosylation (FcA2G2F2). This structure increased from 5.5% in the

pooled healthy sera, to 6.2% in the pooled cirrhotic sera and was 9.6% in the pooled HCC sera. It is noted that these changes were accompanied by decreases in the level of the bi-antennary glycan.

Identification of fucosylated proteins in the depleted proteome

In an effort to identify fucosylated proteins in the HCC seppro-depleted proteome, core and outer arm fucosylated proteins were extracted from the seppro-depleted samples using the lectin AAL as we have done previously²³. Proteins were subsequently digested with trypsin and identified by LC MS/MS. Efforts were not made to quantify using LC MS/MS but rather identified proteins would be further analyzed using traditional lectin-FLISA (lectin-Fluorophore-Linked Immunosorbent Assay) based approaches. Table II shows the list of protein found to be associated with the HCC fuome (fucosylated proteome) following depletion of the major acute phase proteins. Surprisingly, the number of proteins in this list was small and most likely does not fully represent the entire HCC fuome. However, as our main interest is in the discovery of biomarkers, several of these proteins were further examined by Lectin-ELISA techniques to confirm their identification as part of the HCC fuome. A diagram of a typical lectin-FLISA is shown in figure 5A. In this case, an antibody to human hemopexin, which has been modified through mild periodate oxidation is conjugated to bottom of a 96 well plate. The sample is added and fucosylated glycoforms identified using a biotin-conjugated lectin. Bound lectin is then detected using IRDye™ 800 Conjugated streptavidin and the signal intensity measured using the Odyssey® Infrared Imaging System. In all cases, signal intensity was compared to signals detected with commercially purchased human serum (Sigma Chemicals). It is noted that the lectin-FLISA detects the amount of fucosylation present on an equal amount of captured molecules from each patient sample and is performed in a manner that is independent of the total amount of protein in any given patient. Thus, even if proteins levels are different in different individuals, this assay will only measure the relative proportion of altered glycoprotein.

Figure 5B shows a typical Lectin-FLISA confirming the presence of fucosylated hemopexin, fucosylated Alpha-2-HS-glycoprotein (referred to as fetuin-A), fucosylated anti-1-anti-chymotrypsin and fucosylated transferrin in the sera of patients with HCC. As this figure shows, compared to the commercially purchased serum, increased amounts of lectin binding can be observed in the HCC sample following incubation of captured protein with lectin. This is true for the four proteins shown here. In addition, not only are these proteins fucosylated but they also appear to be increased in HCC patients as opposed to patients with cirrhosis, suggesting that they may make good biomarkers of HCC.

Analysis of fucosylated hemopexin and fetuin A in a cohort of 332 patients

Based upon the identification of proteins in the HCC fuome and the confirmation via lectin-FLISA, we analyzed the level of fucosylated hemopexin and fucosylated Fetuin-A in a patient cohort of 332 patients with varying levels of liver disease. The fucosylation of two proteins, hemopexin and fetuin-A, were tested for their ability to detect HCC in a sample group of 332 patients. This cohort consisted of 72 patients with HCC, 33 patients infected with HBV but without cirrhosis, 133 patients infected with HCV, 62 patients with other non-viral liver disease and 20 control patients with no evidence of liver disease. The method

used was the lectin-FLISA as described in figure 5 and the results are presented in figure 6. For both fucosylated hemopexin and fucosylated fetuin-A, relative levels were compared to commercially purchased serum. As figure 6 shows, there was a substantial increase in the level of both fucosylated hemopexin (figure 6A) and fucosylated fetuin A (figure 6B) in patients with HCC. The level of fucosylated hemopexin was 1.4 fold elevated in HCC patients (compared to purchased serum) and ranged from 0.74 to 0.92 fold elevated in all non-HCC patients samples (see figure 6A). Statistical significance was observed between the cirrhosis group and all HCC groups ($P<0.0001$) but not between any of the non-HCC groups ($p>0.05$). Similar alterations were observed with fucosylated fetuin-A, which had a mean increase of 2.5 fold in patients with HCC and ranged from 1.2 to 1.4 fold elevated in all non-HCC patients samples (see figure 6B). As with fucosylated hemopexin, statistical significance was observed between the HCC group and the cirrhosis group ($P<0.0001$) but not between the non-HCC groups ($p>0.05$).

Statistical analysis of fucosylated AAT and fucosylated kininogen

Receiver operator characteristic (ROC) curves were plotted to determine overall performance and to identify the sensitivity and specificity for each marker in differentiating HCC from those without cirrhosis. As shown in figure 7 the AUROC curve for fucosylated hemopexin was 0.9512 with an optimal sensitivity of 92% and a specificity of 92%. Similarly, fucosylated fetuin-A had an AUROC of 0.8691 with a sensitivity of 72% and a specificity of 85%. When examining just HCC versus cirrhosis, fucosylated hemopexin had an AUROC of 0.8665 with a sensitivity of 93% and a specificity of 78%. For fucosylated fetuin-A, when examining HCC versus cirrhosis, the AUROC was 0.8965 with a sensitivity of 76% and a specificity of 75%. The performance of these markers when used in combination was also tested but did not increase the AUROC.

Discussion

We have previously identified several glycoproteins that contained altered fucosylation with the development of HCC^{22, 23}. In an effort to further identify proteins in the fucosylated proteome, we have utilized a method to deplete the serum of several of the major abundant serum proteins prior to analysis via LC MS/MS. Using such a method we could observe changes in glycosylation that were consistent with our previous findings^{22,23}. To determine if any of the proteins identified in the fucosylated proteome could be useful in the diagnosis and clinical management of HCC, we examined the levels of two identified proteins in a 320+ patient study. Patients with HCC generally had higher levels of both fucosylated hemopexin and fucosylated fetuin-A than patients with cirrhosis or other forms of liver disease. Indeed, the performance of these markers, individually and in combination, were impressive and superior to what has been published for the current gold standard for the detection of HCC, AFP.

The core fucosylation of N-linked glycoproteins occurs in the Golgi apparatus²⁵. The exact mechanisms for increased fucosylation in HCC are unknown, but are thought to involve increases in both the levels of the enzyme and the substrates involved in core fucosylation²⁶. Thus it is possible that these markers reflect some alteration in the Golgi apparatus. Indeed,

recent reports have suggested that in regards to the liver, the fucosylation of proteins is involved in cell sorting²⁷. Thus, it is conceivable that the appearance of fucosylated proteins in the serum may reflect a common defect in the Golgi apparatus. This is currently under investigation.

In summary, we have developed a Lectin-FLISA based method for the analysis of fucosylated glycoforms of two secreted liver glycoproteins. These markers when used alone had an overall performance that was better than what has been reported for AFP alone. These data need to be confirmed in larger cohorts of patients to determine if these markers are truly reliable serum marker of early HCC, and to compare its accuracy with AFP in patients of diverse gender, ethnicity, etiologies of liver disease, and to determine its role in HCC surveillance. Future studies should also test the benefit of combinatorial analysis with other potential markers of HCC, such as des-gamma-carboxy prothrombin (DCP).

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Figure 1. Flow Diagram of Glycoproteomic approach

Briefly, 100 μ l of pooled serum samples are fractionated into abundant and less abundant serum proteins using the Seppro IgY Mixed 12 system. Glycan analysis and western blotting are performed to ensure specific extraction and to determine non specific loss. N-linked glycan analysis is used to identify glycan changes that can be targeted with lectin specific enrichment. Proteins within the seppro unbound (less abundant) lectin bound fraction are identified through LC MS/MS.

Figure 2. Depletion of albumin (top), alpha 1 anti-trypsin (middle) and IgG from human serum using the SepproTM MIXED12 system

A) The level of total (T), bound (B) or Seppro unbound (U) protein is shown for each panel. As this figure shows, the vast majority of the albumin, AAT and IgG can be removed using this system. **B)** Identification of non targeted proteins in depleted serum by immunoblot. Top panel shows the presence of AFP in total, bound or unbound samples. Bottom panel shows identical analysis of Apo-D.

Figure 3. De-sialated N-linked glycan profiles of pooled human serum from healthy, cirrhotic or HCC+ individuals after depletion of 12 highly abundant serum proteins

Briefly, Normal phase HPLC N-linked glycan analysis was performed on samples from the Seppro-depleted fraction from either control patients, cirrhotic patients or from HCC patients. Two major glycan structures increase with the development of HCC while one major peak is slightly reduced. These are indicated in the figure. Peak identification was made through sequential exoglycosidase digestion (Data not shown). See text for more detail.

Figure 4. MS/MS spectrum of the hemopexin peptide YLVQGTQVYVFLTK
For spectra, X- axis is M/Z; Y axis is relative abundance. Xcorr = 4.694

Figure 5. Lectin-FLISA for the measurement of fucosylated glycoproteins

A). General Lectin-FLISA methodology. Periodate oxidized mouse anti-human hemopexin was used as the capture antibody and the level of fucosylated protein determined by a biotin conjugated lectin (AAL) and detected using IRDyeTM 800 Conjugated Streptavidin. Signal intensity was measured using the Odyssey® Infrared Imaging System. In all cases sample intensity was compared to commercially purchased human serum (Sigma Chemicals). **B)** Lectin-FLISA for the detection of fucosylated hemopexin, Fetuin A, alpha-1-anti-chymotrypsin or transferrin from either pooled control patients (from 10 individuals), cirrhotic patients (from 10 individuals) or HCC patients (from 10 individuals).

Figure 6. Increase in lectin reactive hemopexin and fetuin-A with the development of HCC
 The level of lectin reactive hemopexin (left panel) or lectin reactive fetuin-A (right panel) in patients with HCC, cirrhosis HBV infection, HCV infection, other liver diseases (OLD) or control patients. The mean value for each group is plotted along with the 95% confidence interval for the mean. For graph, X-axis represents the patient group. Y-axis is fold increase in lectin reactive hemopexin or fetuin A as compared to commercially purchased serum. The *n* value, mean, and standard error are provided for each group below the graph.

Figure 7. Receiver operator characteristic (ROC) curves for the analysis of fucosylated hemopexin (left panel) or fucosylated fetuin-A (right panel)
ROC curves were used to determine the ability to discriminate HCC from non HCC for either fucosylated hemopexin or fucosylated fetuin-A.

Table 1

Patients Utilized in Study

Disease Diagnosis ¹	HCC ²	Cirrhosis ³	HBV ⁴	HCV ⁵	OLD ⁶	Controls ⁷
Number	72	32	33	133	62	20
Etiology% (HBV/HCV/crypto/alcohol/other) ⁸	14/52/6/20/8	N/A	N/A		0/0/15/16/69	N/A
Age	58.04±11	50±8	58.6±12	58±3	51±3	55±8
Gender M:F%	71:29	84:16	75:25	60/40	56:44	50:50
MELD Score ⁹	11.8±5	N/A	N/A	9±2	N/A	N/A
Child Class (A/B/C/) or NA% ¹⁰	52:29:9:10	88:8:4	N/A	N/A	N/A	N/A
Tumor Stage (1/2/3/4) % ¹¹	26:48:12:14	NA	N/A	NA	N/A	N/A

¹ Samples were provided coded from St. Louis University Medical School.

²⁻³ HCC or cirrhosis was determined by MRI or by liver biopsy.

⁴ Patients classified as HBV only were defined as those with HBsAg positivity but no evidence of liver cirrhosis.

⁵ Patients classified as HCV only were defined as those with HCV RNA positivity but no evidence of liver cirrhosis.

⁶ OLD, other liver disease including cryptogenic liver disease, alcohol induced liver disease, Nonalcoholic steatohepatitis (NASH) and autoimmune hepatitis.

⁷ Patients without any evidence of liver disease were used as controls.

⁸ Etiology: HBV, hepatitis B virus; HCV, hepatitis C virus; crypto, cryptogenic liver disease; alcohol, alcohol induced liver disease; other, liver disease of unknown origin.

⁹ MELD: Model for end stage liver disease.

¹⁰ The percent of patients with each Child-Pugh score is given as a percentage in each group.

¹¹ Tumor staging was determined using the United Network of Organ Sharing-modified TNM staging system for HCC. The percent of patients within each stage is given. N/A, not available

Table II

Proteins associated with the Seppro unbound lectin-bound proteome

Protein	Accession	% Coverage	# of unique peptides
Alpha-2-macroglobulin precursor	P01023	16.3%	18
Apolipoprotein B-100 precursor	P04114	4.8%	18
Ceruloplasmin precursor	P00450	20.3%	16
Complement C3 precursor	P01024	12.2%	13
Alpha-1-antichymotrypsin precursor	P01011	34.0%	11
Hemopexin precursor	P02790	27.7%	10
Complement C4 precursor	P01028	7.5%	9
Angiotensinogen precursor [Contains: Angiotensin I	P01019	18.1%	6
Clusterin precursor	P10909	15.6%	6
Complement factor B precursor	P00751	9.9%	6
Lumican precursor	P51884	25.1%	6
Plasma protease C1 inhibitor precursor	P05155	13.6%	6
Kininogen precursor	P01042	9.8%	5
Vitamin D-binding protein precursor	P02774	15.8%	5
Complement C2 precursor	P06681	5.7%	4
Complement factor H precursor	P08603	3.4%	4
Inter-alpha-trypsin inhibitor heavy chain H1 precursor	P19827	5.1%	4
Inter-alpha-trypsin inhibitor heavy chain H4 precursor	Q14624	5.7%	4
Afamin precursor	P43652	6.0%	3
Alpha-1B-glycoprotein precursor	P04217	7.7%	3
Complement C1s component precursor	Q9UCV4	6.0%	3
Insulin-like growth factor binding protein complex	P35858	6.1%	3
L-selectin precursor	P14151	8.9%	3
protein Rei, Bence-Jones	751419	15.4%	3
Carboxypeptidase N 83 kDa chain	P22792	4.7%	2
Carboxypeptidase N catalytic chain precursor	P15169	5.2%	2
Coagulation factor V precursor	P12259	1.0%	2
Complement C5 precursor	P01031	2.1%	2
C-reactive protein precursor	P02741	10.3%	2
Myosin-reactive immunoglobulin kappa chain variable region	Q9UL85	29.6%	2
Neural cell adhesion molecule	O00533	1.9%	2
Thyroxine-binding globulin precursor	P05543	5.1%	2
Vascular cell adhesion protein 1 precursor	P19320	3.5%	2
ADAMTS-1 precursor	Q9UHI8	1.3%	1
Alpha-2-HS-glycoprotein precursor (Fetuin-A)	P02765	2.7%	1
Apolipoprotein B	Q7Z600	0.3%	1
Breast cancer antigen NY-BR-1	Q9BXX3	0.7%	1
CD5 antigen-like precursor	O43866	3.2%	1
complement C7	CAA60121.1	1.8%	1

<u>Protein</u>	<u>Accession</u>	<u>% Coverage</u>	<u># of unique peptides</u>
Complement factor I precursor	P05156	2.2%	1
Dedicator of cytokinesis protein 10	Q96BY6	0.5%	1
Dematin	Q08495	2.7%	1
Fibroblast growth factor-11	Q92914	5.3%	1
Ficolin 3 precursor	O75636	3.0%	1
Fructose-bisphosphate aldolase A	P04075	3.6%	1
Guanylate cyclase soluble, alpha-3 chain	Q02108	1.4%	1
Histidine-rich glycoprotein precursor	P04196	1.7%	1
Insulin-like growth factor binding protein 3 precursor	P17936	2.7%	1
M130 antigen cytoplasmic variant 1 precursor	Q07899	1.5%	1
Mannose-binding protein C precursor	P11226	5.2%	1
MHC class II antigen	Q9BD47	16.9%	1
Plasminogen	AH60513.1	1.2%	1
PRO2266	Q9P176	8.2%	1
Prothrombin precursor	P00734	1.9%	1
Sacsin	Q9NZJ4	0.3%	1
Serum amyloid P component precursor	P02743	5.8%	1