Serum Proteomics and Biomarkers in Hepatocellular Carcinoma and Chronic Liver Disease

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Abstract

Purpose: Proteomic profiling using surface enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF MS) enables the identification of biomarkers for cancer. We evaluated the sensitivity and specificity of SELDI-TOF MS for detection of established hepatocellular cancer (HCC) and compared it against α-fetoprotein (AFP), Lens culinaris agglutinin – reactive α-fetoprotein (AFP-L3), and prothrombin induced by vitamin K absence-II (PIVKA-II).

Experimental Design: Forty-one patients with HCC and 51 patients with hepatitis C cirrhosis were enrolled. Serum was analyzed by SELDI-TOF MS using three Ciphergen protein array types.

Results: An 11-peak algorithm for HCC detection was identified. Using the AFP cutoff of 20 ng/mL, the sensitivity was 73% and the specificity was 71%. Using the AFP-L3 cutoff of 10% yielded a sensitivity of 63% and a specificity of 94%. Using the PIVKA-II cutoff of 125 milliabsorbance units (mAU), the sensitivity was 84% and the specificity was 69%. Overall, the sensitivity and specificity of SELDI-TOF MS for HCC were 79% and 86%, respectively. In multivariate analysis, the 11-peak SELDI profile was predictive of HCC independent of AFP, PIVKA, and AFP-L3. Among eight patients with the largest tumor size of <2 cm, SELDI-TOF MS correctly identified seven whereas AFP, AFP-L3, and PIVKA-II identified only three, one, and one, respectively. One of the 11 peaks in the SELDI-TOF MS 11-peak predictor from SELDI-TOF MS was identified as cystatin C.

Conclusions: SELDI-TOF MS accurately distinguished patients with HCC from those with hepatitis C virus cirrhosis, was more accurate than traditional biomarkers in identifying small tumors, and should be further evaluated.

The incidence of hepatocellular carcinoma (HCC) is on the rise in the United States (1–3). Recently, El-Serag et al. showed that the incidence of HCC had increased from 1.8 per 100,000 to 2.5 per 100,000 over one decade and that nearly all of this increase was attributable to infection with hepatitis C virus (HCV; ref. 2). Once cirrhosis has developed, retrospective studies have suggested that patients will develop either hepatic decompensation or HCC at a rate of 2% to 7% per year (4–8). The general practice among many physicians has been to screen for HCC using ultrasound and serum α-fetoprotein (AFP) levels at 3-month to 6-month intervals. However, even with this screening regimen, many patients still present with either large HCC (>5 cm) or multifocal HCC (more than three lesions) or HCC that has invaded the portal vein or other critical structures. The limitations of ultrasound, the primary radiologic screening modality under current use, include its operator dependence and its poor ability to differentiate malignant from benign nodules in the small cirrhotic liver. Although imaging with triphasic computed tomography scan and magnetic resonance imaging with i.v. gadolinium can improve the diagnostic accuracy, these techniques are time consuming and too expensive for widespread screening at the present time. Because outcomes with HCC are likely to be improved by early detection, better serologic tools for early detection are needed.

The most commonly used serum marker of HCC is AFP. It has a reported sensitivity of 39% to 65% and specificity of 65% to 94% and has multiple limitations when applied to patients with HCV (9–11). Recently, high levels of AFP have been reported in HCV patients with fibrosis and necroinflammation resulting from the natural process of disease progression in HCV and unrelated to HCC (12, 13). In the cohort of patients...
from the Hepatitis C Antiviral Long-term Treatment against Cirrhosis study, 27% of patients with HCV and cirrhosis had an AFP of $\geq 20$ ng/mL in the absence of HCC, and AFP levels declined with antiviral therapy (14).

AFP also seems to have reduced sensitivity for smaller tumors (15), and some authors argue that it has limited utility as a screening test (10). Recently, the American Association for the Study of Liver Diseases guidelines for HCC screening have recognized the overall poor performance of AFP and have removed it from the screening recommendations (16).

Lens culinaris agglutinin–reactive AFP (AFP-L3) and des-γ carboxyprothrombin, also known as prothrombin induced by vitamin K absence-II (PIVKA-II), have also been evaluated as biomarkers for HCC in retrospective longitudinal studies. These biomarkers have been primarily evaluated in patients with established HCC but have not been rigorously evaluated for HCC screening. AFP-L3 measurements in over 2,000 cirrhotic patients that followed between 12 and 72 months identified 71 HCCs with a sensitivity of 61% to 75% and a specificity of 53% to 90% (17–19). PIVKA-II has been studied prospectively and compared with AFP in four studies with 1,138 cirrhotic patients followed for up to 48 months of whom 99 developed HCC (20–23). Determination of PIVKA-II at the time of diagnosis yielded a sensitivity of 23% to 57% and a specificity of 90% in the largest study. Overall, the sensitivity in these studies was no better than for AFP, but PIVKA-II specificity was superior to the 62% reported for AFP.

Proteomic profiling using surface enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF MS) has been evaluated for the ability to identify established HCC (24–26). In addition, SELDI-TOF MS has led to the identification of a new biomarker for HCC, the C-terminal part of the V10 fragment of vitronectin (24). However, SELDI-TOF MS has yet to be applied to a longitudinal cohort of at risk patients for its assessment as a screening tool.

We set out to evaluate the sensitivity and specificity of SELDI-TOF MS for detection of established HCC and to compare its operating characteristics to those of the established biomarkers AFP, AFP-L3, and PIVKA-II.

Materials and Methods

Study population. Two groups of patients were studied. The first group consisted of 41 patients with clinical or biopsy-confirmed cirrhosis complicated by HCC. All patients were prospectively diagnosed with HCC at Beth Israel Deaconess Medical Center. The diagnosis of HCC was confirmed by at least one of the following: (a) histology, (b) new hepatic lesion with an AFP of $>1,000$ ng/mL, and/or (c) new hepatic lesion with arterial phase enhancement on computed tomography or magnetic resonance imaging. The second group included 51 patients with compensated HCV cirrhosis and no HCC. All these patients had biopsies reviewed by a single pathologist and had metavir stage 4 fibrosis and variable degrees of inflammation (median grade of inflammation, 2). The cirrhosis control patients represent a typical HCV population with cirrhosis. The cirrhosis group had at least 2 years of follow up from the time serum was obtained for these studies. The follow up included ultrasound and AFP every 6 months for at least 2 years with no evidence of development of HCC. All samples were stored at -80°C.

Serum biomarker analysis. PIVKA-II was measured by enzyme immunoassay using Etest PIVKA-II kits (Eisai Laboratory) following manufacturer’s instructions. Total AFP (AFP-L1 + AFP-L3) and the percentage of AFP-L3 contents of serum samples were determined using the LiBaSys clinical autoanalyzer (Wako Diagnostics).

Ciphergen ProteinChip SELDI-TOF MS technique. All 92 samples were analyzed by standard SELDI-TOF MS technique described previously (27). All serum samples were analyzed on three different types of protein chips (CM10, IMAC30, and H50) containing cationic, metal chelate, and hydrophobic chromatographic surfaces. The cirrhosis and HCC samples were randomly split into a training set (26 cirrhosis, 20 HCC) and an independent validation set (25 cirrhosis, 19 HCC). For each chip type, individual protein peaks, which represent polypeptides of the same or similar molecular weight, were detected using the Ciphergen Biomarker Wizard software (Ciphergen). We interrogated the serum samples for the full range of protein peaks whose molecular mass lies between 2,000 to 40,000 Da. The serum protein peak data were normalized using the total ion current method as previously described (27). All protein peaks have a numerical value, and thus, no informative peak is missing in any sample. Following manufacturer’s specifications, the normalization step is corrected for the discriminatory peaks from each other, we applied the Genes@Work pattern recognition software (28). To test the prediction accuracy of the discriminatory peaks, we used leave-one-out cross-validation in the GeneCluster software (29). We determined the smallest protein signature with the highest discrimination in the training set and then tested the performance of this protein signature in the independent validation set (30–35). A hierarchical clustering technique was used to construct an unweighed pair group method with arithmetic mean tree using Pearson’s correlation as the metric of similarity (36). This tree represents the differentiating pattern of the predictive peaks on the training set based on the profile observed on the chips.

Enrichment of the 13.391-Da protein marker. A spin column containing Q Ceramic HyperD F beads (Ciphergen) was used to enrich the serum sample for the peak of interest. The column was washed thrice with 50 mmol/L Tris (pH 9). Forty microliters of serum were diluted in 500 μL of 50 mmol/L Tris (pH 9) and incubated for 90 min with the beads in the column rocking at room temperature. The column was centrifuged for 30 s at 800 × g and the flow through recovered. The washings were repeated twice with 150 μL of 50 mmol/L Tris (pH 9) and the flow through pooled and dried down in a SpeedVac centrifuge. The dry pellet was resuspended in 40 μL of nonreducing SDS loading buffer (Boston BioProducts) and subjected to electrophoresis in a 10% polyacrylamide Tris-Tricine gel (Bio-Rad Laboratories). The gel was stained with the Silver Quest kit (Invitrogen), and the piece of gel containing the protein of interest was excised for digestion.

The gel piece was washed for 15 min in 50% acetonitrile before being dried totally in a SpeedVac centrifuge. It was then reduced with 10 mmol/L DTT in 100 mmol/L NH$_4$HCO$_3$ for 30 min at 56°C and alkylated with 55 mmol/L iodoacetamide for 20 min. The gel piece was then repetitively washed with 100 mmol/L NH$_4$HCO$_3$ and dried with acetonitrile. The dried gel was digested by overnight incubation at 37°C with 15 μL of 25 μg/mL trypsin (Promega). The resulting peptides were extracted with 20 mmol/L NH$_4$HCO$_3$ followed by 5% formic acid in 40% acetonitrile.

Identification of the 13.4-KDa marker by tandem mass spectrometry. A 2-μL aliquot of sample was injected onto a microcapillary reverse phase liquid chromatography tandem mass spectrometry system using a self-packed 75 mm i.d. × 10 cm length C$_{18}$ column (New Objective) at a flow rate of 300 nL/min. Data-dependent MS/MS spectra were collected using a ThermoElectron LTQ two-dimensional linear ion trap mass spectrometer operated in the positive ion mode. MS/MS spectra were searched against a reversed nonredundant protein database from PIR-NRREF using the Sequest algorithm. Identified peptide sequences were validated using score cutoffs, as well as manual inspection, to insure that the sequences were consistent with the typical b-series and y-series fragment ions.

Validation of the 13.4-KDa marker by immunodepletion. Ten micro-liters of serum were incubated with 40 μL of polyclonal anti–cystatin C
antibody (Upstate) and 170 μL of PBS for 100 min at room temperature with gentle rocking. Fifty microliters of protein A/protein G coupled to agarose beads (Santa Cruz Biotechnology), preincubated for 15 min in 5 mg/mL bovine albumin, were then added to the serum/antibody mixture, and rocking continued for 2 h at room temperature. The tube was then centrifuged to remove the antigen–antibody–protein A adduct, and the supernatant was analyzed by SELDI-TOF MS.

**Validation of the 13.4-kDa marker by pull down.** Two microliters of anti–cystatin C antibody (0.1 μg/μL) were spotted on an RS100 chip (Ciphergen) for 2 h, followed by a 1-h incubation with 100 mmol/L bovine serum albumin. The chip was then washed with 1× PBS, spotted with 2 μL of the test serum along with 2 μL of 1× PBS, and incubated at room temperature for 2 h in a humid chamber. After incubation, the chip was washed with 50 mmol/L Tris + 1 mol/L urea + 0.1% chaps + 0.5 mol/L NaCl, 1× PBS, and then 5 mmol/L HEPES. The chips were air dried for 5 min, spotted with 50% SPA, and analyzed by SELDI-TOF MS.

**Measurement of serum cystatin C concentration by ELISA.** Cystatin in serum samples was quantified using a human cystatin C ELISA kit (BioVendor) following manufacturer’s instructions. Human cystatin standards were provided in the kit (200-10,000 ng/mL), and test serum samples were diluted 1:400 in the dilution buffer supplied. One hundred microliter aliquots of the diluted standards and test samples were added in duplicate to the wells of a microtiter plate coated with antihuman cystatin C antibody. Dilution buffer alone was added to a pair of duplicate wells to serve as blank. After incubation at room temperature for 30 min on an orbital shaker, the plate was washed thrice with the wash solution and 100 μL of antihuman cystatin antibody labeled with horseradish peroxidase was added to the wells. The plate was incubated for 90 min at room temperature followed by washes as before and addition of 100 μL of substrate solution containing hydrogen peroxide and tetramethylbenzidine to the wells. The plate was covered with aluminum foil to protect from light and incubated for 10 min to allow for color development. The reaction was stopped by the addition of 100 μL of stop solution, and the optical densities were determined by reading absorbance at 450 nm. A standard curve of concentration of Cystatin versus absorbance was plotted using the four-variable function, and the test values were derived from the measured absorbance using this curve. Absorbance measurements were made using a VERSAmax microplate reader equipped with SOFTmax PRO, which enabled automatic calculation of the results.

**Statistical analysis.** The diagnostic accuracy of biomarker tests was determined using the area under the curve of the receiver operating characteristic. Comparisons between groups for ELISA tests was done using one-way ANOVA and unpaired t tests. Multivariate analysis to control for confounding was carried out by logistic regression. A significant value was taken as P < 0.05.

**Results**

**Study population.** Forty-one patients with HCC were identified, all with underlying cirrhosis as determined by biopsy or clinical features. Additional patient characteristics with respect to age, sex, method of diagnosis, serum AFP, largest lesion size, number of lesions, and CLIP score (37) are shown in Table 1. The CLIP score includes the variables of Child Pugh class, tumor morphology uninodular (<50% of liver), multinodular (<50% of liver), massive (>50% of liver), AFP level (<40 or >40 ng/mL), and the presence or absence of macrovascular invasion. Fifty-one patients with compensated HCV cirrhosis were also identified (41 men and 10 women; mean age, 50 years; age range, 23-66 years). A control group of 24 patients with biopsy-proven mild HCV (stage 0/stage 1) was used as the disease control group for ELISA for cystatin C.

**Table 1. Clinical characteristics of 41 patients with HCC used in biomarker and proteomic studies**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age range, y (mean)</td>
<td>43-82 (60)</td>
</tr>
<tr>
<td>Sex (% M/% F)</td>
<td>85:15</td>
</tr>
<tr>
<td>Method of diagnosis</td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td>66%</td>
</tr>
<tr>
<td>Radiology</td>
<td>15%</td>
</tr>
<tr>
<td>Radiology, AFP &gt; 1,000</td>
<td>20%</td>
</tr>
<tr>
<td>Etiology</td>
<td></td>
</tr>
<tr>
<td>Hepatitis C</td>
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</tr>
<tr>
<td>Alcohol</td>
<td>15%</td>
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<tr>
<td>Hepatitis B</td>
<td>12%</td>
</tr>
<tr>
<td>Cryptogenic</td>
<td>5%</td>
</tr>
<tr>
<td>NASH</td>
<td>2%</td>
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<tr>
<td>Hemochromatosis</td>
<td>2%</td>
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<tr>
<td>Child Pugh class</td>
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<tr>
<td>A</td>
<td>46%</td>
</tr>
<tr>
<td>B</td>
<td>39%</td>
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<tr>
<td>C</td>
<td>15%</td>
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<tr>
<td>AFP</td>
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<tr>
<td>&lt;20 μg/L</td>
<td>29%</td>
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<tr>
<td>20-100 μg/L</td>
<td>24%</td>
</tr>
<tr>
<td>&gt;100 μg/L</td>
<td>46%</td>
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<td>Largest lesion size</td>
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<td>21%</td>
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</tr>
<tr>
<td>2-3</td>
<td>34%</td>
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<tr>
<td>4-6</td>
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</table>

**AFP, AFP-L3, and PIVKA-II.** The results for tumor markers in both groups showed a very wide range particularly for AFP. Overall, the mean results for AFP in cirrhotic patients was 35 ng/mL (range, 1-730) versus 19,390 ng/mL (range, 1.3-207,160) in HCC. Similarly, for AFP-L3, the mean was 1.9% (range, 0-13.4%) in cirrhosis versus 28.6% (range, 0-83%) in HCC, whereas for PIVKA-II, the corresponding numbers were 106 mAU (range, 0-2,071) in cirrhosis versus 4,587 mAU (range, 20-24,712) in HCC.

Analysis of the diagnostic accuracy of AFP, AFP-L3, and PIVKA-II was made by receiver operating characteristics (Fig. 1). The areas under the curve (+95% confidence interval) were 0.80 for AFP (0.71-0.90), 0.81 for AFP-L3 (0.71-0.90), and 0.88 for PIVKA-II (0.81-0.95). We used an AFP cutoff of 20 ng/mL because this has been the cutoff suggested for the diagnosis of HCC and also for increased risk of subsequent development of HCC (14, 16). The percentage of patients with varying AFP cutoffs for HCC are shown in Table 1, and in the control population, 37 patients (72%) had AFP of <20 ng/mL, 11 patients (21%) had an AFP between 20 and 100 ng/mL, and 3 patients (7%) had an AFP of >100 ng/mL. Using the AFP cutoff of 20 ng/mL, the sensitivity was 73% (60-85%) and the specificity was 71% (55-84%). Using the AFP-L3 cutoff of 10% yielded a sensitivity of 63% and a specificity of 94%. Using the PIVKA-II cutoff of 125 mAU, the sensitivity was 84% (71-92%) and the specificity was 69% (53-82%). Using the combined criteria of AFP (>20 ng/mL) and an AFP-L3 fraction,
A 10% improvement improved the specificity to 98% but reduced the sensitivity to 59%. The Youden's index (sensitivity + specificity - 1), one estimate of overall test accuracy, was 0.44 for AFP, whereas the corresponding value for AFP-L3 and PIVKA-II were 0.57 and 0.53, respectively.

However, among those patients with HCC but smaller tumor burdens, these serologic markers did substantially worse. Among 20 patients whose tumors satisfied the Milan criteria, with a single tumor of <5 cm or no more than three tumors with the largest at <3 cm, AFP identified 11 (sensitivity, 55%), AFP-L3 identified 8 (sensitivity, 40%), and PIVKA-II identified 9 (sensitivity, 45%). Among eight patients with the largest tumor (<2 cm), AFP identified three (sensitivity, 38%) whereas AFP-L3 and PIVKA-II identified only one each (sensitivity, 13%).

**SELDI-TOF MS.** Among the 41 patients with HCC, sera from two patients did not generate any spectra on one of the three protein chips, and these patients were therefore excluded from subsequent SELDI-TOF MS analysis. The remaining 39 patients with HCC and 51 patients with cirrhosis were included in subsequent analysis by SELDI-TOF MS. Whole serum, rather than serum depleted of high abundant proteins, was used in this analysis, because our experience shows that, within the range of molecular weights of proteins detected by SELDI-TOF, depletion of albumin and other high abundant proteins does not dramatically change the pattern and level of low molecular weight proteins detected by SELDI-TOF. Furthermore, depletion of large number of serum proteins adds additional steps that may reduce reproducibility from one sample to the other. The majority of the SELDI-TOF papers published thus far have used whole serum as a reliable and reproducible approach to identify biomarkers. In aggregate, SELDI-TOF MS detected a total of 1,145 protein peaks on all samples above a signal to noise ratio of 2:1. Three hundred seventy-eight were detected on CM10, 394 were detected on H50, and 373 were detected on IMAC30 protein chips. The cirrhosis and HCC samples were randomly split into a training set (26 cirrhosis, 20 HCC) and an independent validation set (25 cirrhosis, 19 HCC). We first found 261 peaks that differentiated the HCC samples from the cirrhosis samples in the training set. This set of discriminating peaks was then further refined to an 11-peak protein signature with the best prediction performance derived from the training set. The 11-peak protein signature, which contained three proteins from the CM10 chip, four proteins from the IMAC30 chip, and four proteins from the H50 chip, distinguished between cirrhosis and HCC, with a sensitivity of 85%, a specificity of 85% and a Youden's index of 0.7 in the training set.

In Fig. 2, we show the molecular weights of these 11 biomarkers and the heatmap for the training samples using the 11-peak predictor. Complete peak intensity values for all the samples, along with the list of discriminating peaks, predictive signature, and prediction results, can be found in online supplementary data.

To determine the diagnostic performance of the 11-protein signature, we tested this protein profile in an independent validation set of 25 cirrhosis and 19 HCC patients. The validation set confirmed the performance of our protein signature with sensitivity of 74%, specificity of 88%, and Youden's index of 0.62, which was higher than the Youden's index for any of the individual biomarkers.

As with AFP, AFP-L3, and PIVKA-II, we did subgroup analysis for SELDI-TOF. SELDI-TOF MS correctly identified 13 of 19 patients with HCC satisfying the Milan criteria (sensitivity, 68%), including six of eight in the training set and 7 of 11 in the validation set. SELDI-TOF MS also correctly identified seven of eight patients with largest tumor size of <2 cm (sensitivity, 88%), including three of three in the training set and four of five of the validation set. In addition, SELDI-TOF MS correctly identified 18 of 24 patients with cancer related to HCV, including 10 of 13 in the training set and 8 of 11 in the validation set.

**Multivariate analysis for other diagnostic variables.** To determine whether the SELDI profile was predictive of HCC...
independently of other established or emerging diagnostic markers, we did logistic regression analysis. In a multivariate model using SELDI labels, AFP (at 20 ng/mL cutoff), AFP-L3 fraction (at 10% cutoff), and PIVKA-II (at 125 mAU cutoff), SELDI, AFP-L3, and PIVKA-II, were all independent predictors of HCC whereas AFP was not. SELDI labels predicted HCC with an odds ratio of 14.4 (P < 0.001) when adjusted for AFP-L3 AND PIVKA-II. The odds ratios for AFP-L3 and PIVKA-II were 14.1 (P = 0.003) and 7 (P = 0.006), respectively.

Combination of SELDI-TOF MS, AFP, AFP-L3, and PIVKA-II. Recognizing the limitations of each individual test, we next assessed the ability of SELDI-TOF MS, in conjunction with other serologic tests, to diagnose HCC. We used three criteria for the diagnosis of HCC. (a) AFP of >20 ng/mL with an AFP-L3 fraction of >10%, (b) PIVKA-II of >125 mAU, and (c) SELDI-TOF MS categorization as HCC or cirrhosis. We then applied these criteria to the 39 patients with adequate spectra by SELDI-TOF MS. At least one of these three tests was positive in 37 of 39 patients with HCC (sensitivity, 95%), whereas the sensitivity of at least two positive tests was 72% with a specificity of 96%. These results were similar in both the training and validation sets.

Identification of cystatin C as 1 of the 11 biomarkers differentiating between hepatocellular carcinoma and liver cirrhosis. To identify 1 of the 11 biomarkers of our protein signature, we selected the 13,391-Da peak for further evaluation. This peak was chosen due to the relatively high mass spectrometric peak height, clear separation from other peaks, and strikingly different expression in cirrhosis versus HCC patients. Serum from one patient with HCC was fractionated on Q Ceramic HyperD F beads, and the pH 9.0 fraction was loaded on a SDS-polyacrylamide gel. After silver staining of the gel, the band around 14,000 Da was excised and digested by trypsin (Promega) for identification by mass spectrometry of the 13,391-Da protein peak. Using this approach, we were able to purify and identify one of the peaks at 13.4 kDa from the 11-protein signature. Throughout the purification process, the accuracy was followed by comparing the molecular weight of the purified protein by SELDI-TOF MS to the original profile. Using the Thermo Finnigan LTQ ion trap mass spectrometer, we identified three proteins in a gel slice cut out from the region around 13 to 15 kDa containing the protein with the expected molecular weight. The expected molecular weight of one of the identified proteins, cystatin C, closely matched the observed molecular weight of one of the HCC biomarkers. Preliminary data exists that cystatin C may be elevated in liver cirrhosis (38).

Validation by immunodepletion and pull down. To validate that cystatin C was the correct 13,391 Da protein, we did two mass spectrometric immunoassays with a polyclonal antibody against cystatin C. We first incubated an aliquot of serum from an HCC patient with the immobilized anti-cystatin C antibody or a control antibody to bind and deplete cystatin C from the serum and then analyzed the flow through by SELDI-TOF MS. In the anti-cystatin C antibody incubated sample, the expected peak was completely depleted, whereas in the control incubated sample, the expected peak remained, confirming that the peak of interest is cystatin C (Fig. 3).

As a second assay to validate the identity of cystatin C, we immobilized anti-cystatin C antibody on the RS-100 Ciphergen ProteinChip. Uncoated RS-100 chips and chips coated with antibody against nuclear factor-κB were each used as controls. The protein chips were incubated with serum and then extensively washed and analyzed by SELDI-TOF MS to determine which proteins were retained by the anti-cystatin C antibody. The expected protein peak at 13.4 kDa, identical to the molecular weight observed in our 11 protein signature, was precipitated only with the anti-cystatin C antibody, but not with the anti-nuclear factor-κB antibody and uncoated controls (Fig. 4). This further confirms the identity of this peak as cystatin C.

Cystatin C ELISA in serum from HCV and HCC patients. To evaluate whether cystatin C levels were elevated in serum, an ELISA was done on serum from 24 patients with mild HCV (fibrosis score, 0/1), 51 patients with cirrhosis, and 41 patients with HCC. The cystatin C levels were 1,009 + 107 ng/mL.
(P < 0.0004 versus cirrhosis), 1,430 + 57 ng/mL (P < 0.0017 versus HCC), and 1,843 + 125 ng/mL, respectively (one-way ANOVA P < 0.0001). Because cystatin C can be elevated in renal disease, the creatinine was evaluated for all patient groups, and the mean (+95% confidence interval) was 0.85 (+0.13) among patients with mild HCV, 0.80 (+0.08) among patients with HCV cirrhosis, and 0.978 (+0.09) among patients with HCC (one-way ANOVA P = 0.015). Plotting a receiver operating characteristic curve for cirrhosis and HCC yielded an area under the curve of 0.663 (95% confidence interval, 0.536-0.789), which, although not highly diagnostic for a single biomarker, is within an excellent range for this protein as part of the proteomic profile.

**Discussion**

The need for better tests for the diagnosis and screening of patients at risk of HCC is an important target for clinical researchers and one highlighted in the recent NIH roadmap for research in liver diseases (39). In developing a tool for screening, five stages of development of cancer biomarkers have been identified. These stages include preclinical screening where potential biomarkers are identified and clinical assay validation. These first two phases are necessary before progressing to retrospective longitudinal, prospective screening, and case control studies.

This study was focused as a stage 2 screening study to assess the efficacy of a proteomic approach to diagnosing HCC using SELDI-TOF MS. Proteomics represents a potentially powerful tool for the serologic recognition of protein profiles associated with cancer. SELDI-TOF MS may by itself only be used as a discovery tool followed by development of antibody-based multiplex ELISA for diagnostic purposes; alternatively, subtle technological modifications of the mass spectrometer combined with automatic data analysis may convert SELDI-TOF MS into a diagnostic tool itself. We also set out to compare the accuracy of this approach with the accuracy of the previously described biomarkers AFP, AFP-L3, and PIVKA-II, and to determine whether combining these approaches could improve the overall diagnostic efficiency. Finally, in preparation for retrospective screening studies, we needed to identify whether these tests were sensitive to smaller tumors, a characteristic that may be important before evaluating these tests for screening purposes.

SELDI-TOF had an overall sensitivity of 79% and specificity of 86% for diagnosing HCC, which was comparable, or even superior, to any individual or combined biomarker panel. Use of the protein signature from the training set resulted in high sensitivity and specificity in the validation set supporting the diagnostic potential of the SELDI-TOF MS approach.

Among all patients, SELDI-TOF MS did somewhat better than the other markers, but all markers did reasonably well. AFP in the 20% of patients with a new lesion and a level above 100 ng/mL is completely diagnostic. However, the most notable false-negative rate for AFP, AFP-L3, and PIVKA-II came among the eight patients with the lowest tumor burdens — those with the largest tumor of <2 cm — where AFP identified 38% and AFP-L3 and PIVKA-II identified only 13% each. Among these same eight patients, SELDI-TOF MS correctly identified HCC in 88%. Thus, the sharp decline in sensitivity among those with low tumor burdens seen for AFP, AFP-L3, and PIVKA-II was not seen for SELDI-TOF MS. One possible explanation for this is that SELDI-TOF MS is detecting some protein peaks that characterize early HCC. We feel this has important implications for their utility as screening tools, wherein identification of patients with low tumor burden is
essential. Importantly, SELDI-TOF profile was an independent predictor of HCC, even when taking into account other prognostic variables, such as AFP-L3 and PIVKA.

In an effort to improve on the overall sensitivity of these serologic tests, we combined three criteria: (a) AFP of >20 ng/mL with an AFP-L3 fraction of >10%, (b) PIVKA-II of >125 mAU, and (c) SELDI-TOF MS categorization as HCC or cirrhosis. The sensitivity for at least one positive test was 95% with a specificity of 73%, which supports the hypothesis that it may be possible to combine multiple biomarkers to enhance sensitivity while still maintaining acceptable specificity.

Our study has several limitations. First, although all patients in this study had underlying cirrhosis, the non-HCC control group all had HCV as the underlying cause whereas the cohort with HCC had a variety of etiologies for cirrhosis, including HCV, HBV, and alcohol. In addition, all patients in the cirrhosis group had compensated cirrhosis, whereas 15% of the patients with HCC had decompensated disease with Child class C. Future studies should include a more diverse control group and include patients with decompensated disease. However, SELDI-TOF MS did similarly well in identifying HCC across all groups, including those with HCC from underlying HCV cirrhosis and those with compensated cirrhosis, although the numbers in each subgroup were small. Finally, although SELDI-TOF MS did very well among small tumors, we recognize that the number of patients in this subgroup is low.

At least three other articles have addressed the accuracy of SELDI-TOF MS for identification of HCC. Poon identified HCC with 92% sensitivity and 90% specificity, although they did not divide their patients in a training set and an independent validation set (25). Paradis identified HCC with a sensitivity of 85% and a specificity of 91% compared with cirrhotic controls and further identified the most predictive peak to be the C-terminal part of the V10 fragment of vitronectin. Schweger used SELDI-TOF MS profiling to identify 38 differentially expressed proteins in HCC patients and then analyzed a subset of HCC associated with HCV (26). For distinguishing chronic HCV from HCV-HCC, a sensitivity of 61% and a specificity of 76% were obtained. Interestingly, these investigators then combined the SELDI-TOF MS peak values with AFP, PIVKA-II, and GP73 and increased sensitivity to 75% and specificity to 92%.

Our findings are in general agreement with those reported by previous authors, thus providing additional confirmation that a proteomic approach can accurately identify clinical HCC in patients with cirrhosis. We also show a potential advantage of SELDI-TOF MS, which is the ability to detect small HCCs. This may have important implications for its utility in screening for HCC. Finally, we have identified cystatin C as one of the markers in our 11-protein signature, which is overexpressed in HCC, and showed that serum cystatin C levels differ between patients with HCC compared with those with HCV cirrhosis. We conclude that SELDI-TOF MS should be applied to a cohort of at-risk patients for prospective assessment as a screening tool.

Furthermore, more in-depth analysis of the serum proteome upon depletion of the most abundant serum proteins combined with fractionation is likely to provide additional diagnostic markers that will increase both sensitivity and specificity.

Acknowledgments

We thank Shaker Kolia for technical support.

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