Proteomic profiling of serum is an emerging technique to identify new biomarkers indicative of disease severity and progression. The objective of our study was to assess the use of surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) to identify multiple serum protein biomarkers for detection of liver disease progression to hepatocellular carcinoma (HCC). A cohort of 170 serum samples obtained from subjects in the United States with no liver disease (n = 39), liver diseases not associated with cirrhosis (n = 36), cirrhosis (n = 38), or HCC (n = 57) were applied to metal affinity protein chips for protein profiling by SELDI-TOF MS. Across the four test groups, 38 differentially expressed proteins were used to generate multiple decision classification trees to distinguish the known disease states. Analysis of a subset of samples with only hepatitis C virus (HCV)-related disease was emphasized. The serum protein profiles of control patients were readily distinguished from each HCV-associated disease state. Two-way comparisons of chronic hepatitis C, HCV cirrhosis, or HCV-HCC versus healthy had a sensitivity/specificity range of 74% to 95%. For distinguishing chronic HCV from HCV-HCC, a sensitivity of 61% and a specificity of 76% were obtained. However, when the values of known serum markers α-fetoprotein, des-gamma carboxyprothrombin, and GP73 were combined with the SELDI peak values, the sensitivity and specificity improved to 75% and 92%, respectively. In conclusion, SELDI-TOF MS serum profiling is able to distinguish HCC from liver disease before cirrhosis as well as cirrhosis, especially in patients with HCV infection compared with other etiologies. (HEPATOLOGY 2005;41:634-642.)

The incidence of hepatocellular carcinoma (HCC) continues to increase in the United States, while, unfortunately, patient survival with HCC has only marginally improved over the last 20 years. Between 1981 and 1998, the 5-year survival rate only rose from 2% to 5%.2 The poor survival rate is in part related to the diagnosis of HCC at advanced stages, where effective therapies are lacking.3 Surveillance of patients at the highest risk for developing HCC (i.e., patients with cirrhosis) is an important strategy that can potentially decrease the cancer-related mortality rate. Although HCC meets the criteria of a tumor that would benefit from a surveillance program, the poor sensitivity and specificity of currently available tools has prevented widespread implementation of HCC surveillance. For example, α-fetoprotein (AFP) has been the serum marker that is most widely used for diagnosis as well as surveillance of HCC.4,5 However, AFP levels may be normal in up to 40% of patients with HCC, particularly during the early stages (low sensitivity).6 Furthermore, elevated AFP levels may be seen in patients with cirrhosis or exacerbations of chronic hepatitis (low specificity).7 Prospective studies evaluating the performance characteristics of AFP for HCC surveillance...
reported sensitivities of 39% to 64%, specificities of 76% to 91%, and positive predictive values of 9% to 32%. Abdominal ultrasound is the most common imaging modality used in the surveillance of HCC, and it can lead to increased survival of patients with cirrhosis who develop HCC by allowing earlier disease management strategies. The sensitivity, specificity, and positive predictive value with ultrasound have been reported to be 71% to 78%, 90% to 93%, and 14% to 73%, respectively. However, the accuracy of ultrasound can be limited by the ability of the operator and the ability to differentiate HCC from nonneoplastic lesions such as regenerative nodules. Therefore, there is a need for additional serum markers that will improve the detection rate of early HCC.

One serum marker being evaluated, des-gamma carboxyprothrombin (DCP), or prothrombin induced by vitamin K absence-II, is an abnormal prothrombin protein that is increased in the serum of patients with HCC. In a previous cross-sectional study, DCP was shown to be better than AFP in differentiating HCC from nonmalignant chronic liver disease in serum obtained from patients in the United States. A DCP value above 125 mAU/mL among patients with underlying chronic liver disease was associated with a high probability of HCC (sensitivity and specificity ~90%). Furthermore, high DCP levels in serum or expression in late-stage tumor tissues have been linked as poor prognostic indicators for patients with HCC. GP73 is a novel type II Golgi membrane protein of unknown function that is expressed in the hepatocytes of patients with adult giant-cell hepatitis. Increased expression of GP73 in hepatocytes appears to be a general feature of advanced liver disease.

Proteomic techniques are increasingly being applied to identify biomarkers in serum indicative of advanced liver disease, particularly hepatitis and HCC. One of the recent technological advances in proteomics is the surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS). Applications of this technology to clinical fluids have suggested great potential for the early detection of prostate, breast, head-neck, ovarian, pancreatic, bladder, and liver cancers. Recently, SELDI-TOF MS has been successfully used at six separate institutions to reproducibly generate identical protein profiling spectra for quality control sera, and correctly distinguish healthy from prostate cancer subjects based on serum protein profiles. Using similar experimental protocols standardized in this multi-institution study, the objective of the phase I (exploratory) cancer biomarker study reported herein was to determine if protein profiling using SELDI-TOF MS could accurately distinguish patients with different stages of liver disease, especially those associated with hepatitis C virus (HCV) infections ranging from chronic hepatitis to HCV-associated HCC. An initial comparison of the performance of other serum markers (AFP, DCP, GP73) singly, grouped, or in combination with the SELDI protein peaks is also presented.

**Patients and Methods**

**Patient Specimens.** All patients were enrolled from the liver and liver transplantation clinics at the University of Michigan Medical Center between September 2001 and May 2002 with Institutional Review Board approval. Written informed consent was obtained from each patient. Four groups of consecutive subjects were enrolled. One group included subjects with no history of liver disease and normal liver biochemistry, no risk factors for viral hepatitis, and alcohol consumption less than 40 g/wk. The second group consisted of subjects with histologically confirmed chronic hepatitis. The third group consisted of patients with histologically proven cirrhosis and compensated liver disease (Child-Turcotte-Pugh score < 7). A fourth group consisted of patients with histologically proven HCC (Table 1). A 20-mL blood sample was drawn from each subject for AFP and DCP testing more than 2 weeks after liver biopsy was performed. Blood samples were spun and serum was aliquoted and stored at −80°C until testing. Each sample used for proteomic profiling had not been thawed more than once. Blood samples from HCC subjects were drawn before initiation of treatment.

**Serum Liver Biomarker Assays.** AFP was tested using commercially available immunometric assays using enhanced chemiluminescence at the University of Michigan Hospital Clinical Diagnostic Laboratory. The upper limit of normal was 8 ng/mL. DCP levels were measured using an enzyme-linked immunosorbent assay kit (Eitest PIVKA-II; Eisai Co., Tokyo, Japan) per the manufacturer’s instructions and were performed in duplicate. Levels of GP73 in serum were determined via Western blot analysis. Equal volumes of patient sera (0.5 μL/lane) were separated via SDS-PAGE on 4% to 20% polyacrylamide gradient gels. For normalization, each gel also included a lane containing 0.5 μL of serum from a pool of sources negative for HCV and hepatitis B virus (Sigma, St. Louis, MO). GP73-specific signals from the 73-kd species were quantified from X ray film using an AlphaInnotech FluorChem CCD camera with AlphaEase spot densitometry software (both from Alpha Innotech Corp., San Leandro, CA) and were expressed as integrated intensity units relative to the GP73 signal detected in Sigma control serum standard. Values were calculated as the
Table 1. Demographic Information and Etiology of Liver Disease

|                     | Group 1 (n = 39) | Group 2 (n = 36) | Group 3 (n = 38) | Group 4 (n = 57) | P Value (α > .05) |
|---------------------|------------------|-----------------|-----------------|-----------------|-----------------|
| Sex (Male/Female)   | 21:18            | 20:16           | 22:16           | 35:22           | NS              |
| Age (yr)            | 51 ± 11          | 50 ± 6          | 52 ± 8          | 54 ± 13         | NS              |
| Ethnicity (%)       | 90/10/0/0        | 78/16/4/2       | 69/11/9/11      | 88/6/4/2        | NS              |
| Etiology (%)        |                  |                 |                 |                 |                 |
| HCV                 | 75               | 58              | 49              |                 | <.001*          |
| HBV                 | 10               | 11              | 4               |                 | NS              |
| Alcohol             | 0                | 2               | 13              |                 | <.001†          |
| Autoimmune          | 6                | 4               | 2               |                 | NS              |
| Cryptogenic         | 6                | 20              | 26              |                 | .01*            |
| Other               | 3                | 5               | 6               |                 | NS              |
| MELD score          | 4 ± 0.7          | 5 ± 0.8         | 7.2 ± 1.3       | 8 ± 2.3         | <.03‡           |
| ALT (IU/mL)         | 28.6 ± 9         | 67 ± 41         | 112 ± 124       | 81 ± 49         | <.001*          |
| AST (IU/mL)         | 22 ± 5           | 53 ± 36         | 94 ± 85         | 109 ± 59        | .003‡           |
| Bilirubin (mg/dL)   | 0.4 ± 0.2        | 0.5 ± 0.4       | 0.9 ± 0.6       | 1.2 ± 0.9       | .13             |
| AFP (ng/mL)         | 2.94 ± 1.6       | 10.8 ± 23       | 19.7 ± 38       | 11,788 ± 60,359 | .003†           |
| % <20               | 100              | 88              | 77              | 55              |                 |
| % 20-200            | 0                | 12              | 23              | 24              |                 |
| % >200              | 0                | 0               | 0               | 21              |                 |
| DCP (mAU/mL)        | 25 ± 4           | 31 ± 8          | 36 ± 12         | 1,925 ± 235     | <.001†          |
| GP73 (mAU/mL)       | 5.1 ± 3.4        | 6.9 ± 5         | 10.3 ± 9        | 16.6 ± 8        | .001†           |
| TNM stage % (I/II/III/IV) | NA          | NA              | NA              | 16/22/21/13     |                 |

NOTE. All data are presented as the mean ± SD.
Abbreviations: NS, nonsignificant; MELD, model for end-stage liver disease; NHW, non-Hispanic white; AA, African American; H, Hispanic; HBV, hepatitis B virus; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TNM, primary tumor/lymph node/distant metastasis; NA, not applicable.

*Groups 3 and 4 versus groups 1 and 2.
†Group 4 versus groups 1, 2, and 3.
‡Group 4 versus groups 1 and 2.

mean of duplicate or triplicate determinations for each serum sample.

**SELDI Processing of Serum Samples.** Aliquots of each serum specimen at the University of Michigan Medical Center were mixed in a 2:3 ratio of serum to 8 mol/L urea, 1% CHAPS, and were frozen at −80°C before being shipped to the Center for Biomedical Proteomics at Eastern Virginia Medical School. Serum samples were processed robotically on a Biomek 2000 liquid handling system (Beckman Coulter, Fullerton, CA) in a 96-well format for SELDI analysis in the following manner: A further dilution (1:5) of the thawed serum in the 8-mol/L urea buffer was made in 1 mol/L urea, 0.125% CHAPS, and phosphate-buffered saline. Diluted serum was randomly spotted in duplicate onto copper-coated immobilized metal affinity capture (IMAC-Cu) protein chips (Ciphergen Biosystems, Fremont, CA) for SELDI-TOF analysis with the aid of a 96-well bioprocessor. The sample was allowed to bind to the protein chips for 30 minutes at room temperature, followed by washes of phosphate-buffered saline and water. The IMAC-Cu chip arrays were allowed to air dry and a saturated solution of sinapinic acid in 50% (vol/vol) acetonitrile, 0.5% (vol/vol) trifluoroacetic acid was added to each spot.

**SELDI Data Analysis.** The protein chip arrays were analyzed using the SELDI ProteinChip System (PBS-II; Ciphergen Biosystems). The spectra were generated by the accumulation of 192 shots at laser intensity 220 in a positive mode. The protein masses were calibrated externally using purified peptide standards. Spectra were analyzed with Ciphergen ProteinChip software (version 3.1) and normalized using total ion current. Peak clustering in the 1.5- to 20-kd range was performed using Biomarker Wizard Software (Ciphergen Biosystems) at settings that provide a 5% minimum peak threshold, 0.2% mass window, and 2% to 3% signal/noise determination. Intensity values for each peak were then averaged for each duplicate sample pair analyzed and input into BioMarker Patterns software (Ciphergen Biosystems) for classification tree analysis as described previously.29,30,36,37 Briefly, classification trees split the data into two nodes, using one rule at a time in the form of a question. The splitting decisions in this case were based on the normalized intensity levels of peaks from the SELDI protein expression profile. Each peak or cluster identified from the SELDI profile is therefore a variable in the classification process. The process of splitting is continued until terminal nodes are reached and further splitting has no gain in data classification. Multiple classification trees were generated using this process, and the best performing tree was chosen for testing. During the analysis, a pruning step occurs in which branches are removed and the cost of the removal is de-
determined to establish a minimal tree size. This is referred to as a “learning set.” Second, the decision tree was subjected to cross-validation. In this step, the data is partitioned such that randomly selected samples are categorized with the decision tree being tested to ensure that the decision tree is valid. Only these cross-validated values are presented in the data tables herein. The nine SELDI peaks that formed the main splitters of the tree(s) with the highest prediction rates in the cross-validation analysis were selected for further analysis with the different serum markers.

**Statistical Analysis.** Specificity was calculated as the ratio of the number of negative samples correctly classified to the total number of true negative samples. Sensitivity was calculated as the ratio of the number of correctly classified diseased samples to the total number of diseased samples. Comparison of relative peak intensity levels between groups was calculated using the Student $t$ test.

**Results**

**Sample Processing and SELDI Analysis.** The SELDI-TOF approach was applied to 170 patients as described in Table 1. A subset with only HCV-related disease was identified: HCV-HCC ($n = 28$), HCV cirrhosis ($n = 22$), chronic HCV ($n = 27$). Each serum sample was applied to copper-coated immobilized metal affinity chips (IMAC-Cu) in duplicate. All sample loading, processing and analysis steps were fully automated to minimize sample processing errors. Following baseline subtraction and normalization using total ion current, peaks present in all of the samples were labeled and clustered. The peak intensity values of 39 differentially expressed peaks identified in all samples in the 1.5- to 20-kd mass ranges were used for further analyses.

**Comparison of SELDI Spectra.** A gel-view representation of five sample spectra from four groups (healthy, HCV–no cirrhosis, HCV-cirrhosis, and HCV-HCC) in the 5,000- to 12,000-Da range is presented in Fig. 1. At four different distinguishing mass values, a box is drawn to illustrate the differences in intensities for a given peak (5,808, 8,939, 9,501, 11,735 m/z). Scatter plots of the intensities of these same four peaks in all of the HCV-related samples analyzed are shown in Fig. 2. Using the mean intensities of each sample as indicated by the bar, the proteins represented by the 5,808 and 11,735 m/z peaks increased with disease severity. The levels of the 8,939 m/z protein were also increased following HCV infection and were actually highest in the serum from HCV-associated cirrhosis subjects. Conversely, the intensity of the 9,501 m/z protein decreased in all HCV-associated serum samples relative to the healthy subjects.

Table 2 shows the $P$ values of the mean intensities of each of the four peaks for six pairwise comparisons between the different patient groups. When comparing normal samples to liver diseases, the $P$ values decreased with disease severity. The changes were variable when comparing differences among chronic hepatitis, cirrhosis, and HCC samples, although the 5.8 and 11.7 markers were able to distinguish HCV patients with cirrhosis from HCV-associated HCC. The cumulative data illustrate how changes in multiple biomarker proteins can be used as fingerprint patterns reflective of disease state, even though any single protein would not be sufficient for classification.

**Decision Classification Tree Analysis.** For each sample analyzed, intensity values for each peak in the 1.5- to 20-kd range were averaged for duplicate samples and input into the BioMarker Patterns software (Ciphergen Biosystems) for classification tree analysis as described in Patients and Methods. The classification trees split the data into two nodes, using one rule at a time in the form of a question. The splitting decisions in this case were based on the normalized intensity levels of 39 shared peaks from the SELDI protein expression profile across each sample, such that each peak was used as a variable in the classification process. An internal 1/10 sample exclusion, cross-validation process was done automatically for each decision tree generated, and the most significant and best performing tree for each condition was chosen. A separate blinded sample set of sera from healthy individuals ($n = 42$) and those with HCC ($n = 56$) (Table 3) was also evaluated for the validation of the classification trees.
The clinical characteristics of these sera were analogous to those described in Table 1 for the learning set sera.

Initially, serum profiles from healthy subjects were compared individually with serum profiles generated from either non-cirrhosis, cirrhosis, or HCC samples, including the subset of HCV-infected samples (Table 4). A representative diagram of a decision tree for the comparison of all HCC samples versus healthy patients is shown in Fig. 3, the result corresponding to group III.A. in Table 4. A similar process was used to generate a decision tree for each sample pair presented in Tables 4 and 5. Only the cross-validation results for each decision classification analysis are presented. Following generation of the SELDI spectra for the blinded sera on the IMAC-Cu protein chips, the obtained peak clusters were applied to the previously optimized decision tree (Fig. 3). A correct classification of 91% (51 of 56) for HCC and 76% (33 of 42) for healthy patients was obtained.

The results in Table 4 indicate that the SELDI peak profiles were progressively more effective at distinguishing normal samples from non-cirrhosis, cirrhosis, and HCC conditions as the severity of the disease increased, regardless of etiology. A separate stratification of these samples was performed based only on an HCV-associated etiology and analyzed separately. The sensitivities of HCV-associated diseases versus normal sera were better compared when all other liver diseases were combined, as presented in Table 4 (subgroups I.B., II.B., and III.B.). For example, correct classification of HCV-cirrhosis conditions increased to 91% compared with 72% in the cirrhosis sample set that included other types of liver disease.

The ability to correctly classify the more clinically relevant scenarios for surveillance of HCV disease progression from chronic hepatitis to cirrhosis to HCC was examined using the HCV disease stratified sample set used in the initial training set. Chronic HCV samples could be distinguished from HCV-HCC samples with a sensitivity of 71% and specificity of 64% (Table 5). When both chronic hepatitis and cirrhosis samples were combined and compared with HCV-HCC samples, sensitivity decreased to 61%, but specificity increased to 76% (Table 5). To test whether the accuracy of classification could be increased by including clinical data in the analysis, values for AFP, DCP, and GP73 were included in the classification decision tree analysis with all of the SELDI peaks. These values were considered by the algorithm to be additional “peaks” to be used with those from the SELDI analyses to build classification trees. As seen in Table 5, inclusion of these marker protein values increased the correct classification of disease states to 79%/86%.

Fig. 2. Expression level of the (A) 5,808, (B) 8,936, (C) 9,509, and (D) 11,707 m/z proteins for each sample in all of the indicated HCV disease-stratified sample sets. Black bars indicate mean normalized intensity; open circles represent values of individual samples. HCV, hepatitis C virus infection but no cirrhosis; HCV cirr, HCV-associated cirrhosis; HCC, HCV-associated hepatocellular carcinoma.
and 75%/92% sensitivity/specificity, respectively, for both sample sets.

**Discussion**

Proteomic analysis of tissue or serum derived from HCC subjects is an emerging technique for the identification of biomarkers indicative of disease severity and progression.\(^{19-21,38}\) To date, new HCC biomarkers have been sought primarily by using differential two-dimensional gel separations of tumor tissues\(^{38}\) or serum\(^{19,21}\) together with mass spectrometry for protein identification. This is a productive discovery approach for identifying biomarker proteins of masses greater than 10 kd; however, the low sample throughput limits the development of direct diagnostic assays. Use of mass spectrometry proteomic profiling approaches, typified by matrix-assisted laser desorption/ionization and SELDI instrumentation, allows greater sample throughput and the ability to examine low mass proteins (<10 kd) that have been difficult to assay effectively with other methods. Using a clinically defined serum sample set reflective of liver disease progression to HCC, we have obtained results that support the potential use of SELDI-TOF profiling as a surveillance tool to follow disease progression. We also show that known serum markers or other clinical test data can be incorporated into the SELDI peak analysis. The SELDI analysis was comparable to the performance of AFP, the only clinically used marker in this pilot study. Combining the SELDI-derived protein peaks with known serum marker data has also been reported to increase correct classification of a pancreatic cancer serum cohort.\(^{33}\)

Another HCC serum proteomics study using SELDI and immobilized metal affinity protein chips has been reported by Poon et al.\(^{20}\) The serum samples in this study were initially prefractionated into six separate components before application to the copper affinity chips (or a weak cation chip), and a neural network type cluster analysis algorithm was used for classification following peak selection. The best sensitivities and specificities of 92% and 90%, respectively, were obtained for distinguishing chronic liver diseases (n = 20) from late-stage HCC (III/IV, n = 24) samples. Our study differs significantly in that more early-stage HCC samples (I/II) were evaluated, no prefractionation of serum was done, a larger sample size, and the clinical serum set analyzed was more fully stratified for the different liver diseases. Use of a decision tree classification algorithm further enhanced our studies by allowing the inclusion of other serum marker data. Even though two distinct sample preparation and algo-

**Table 2. Comparisons of P Values for the Indicated Markers in Different Sample Pairs**

| Marker (m/z) | Normal vs. HCV | Normal vs. HCV-Cirrhosis | Normal vs. HCC | HCV vs. HCV-Cirrhosis | HCV vs. HCC | HCV Cirrhosis vs. HCC |
|-------------|----------------|--------------------------|---------------|-----------------------|-------------|----------------------|
| 5.8         | 0.72           | <5 × 10^-5               | <5 × 10^-5    | 0.077                 | <5 × 10^-5  | 0.002                |
| 8.9         | 0.0002         | <5 × 10^-5               | <5 × 10^-5    | 0.109                 | 0.91        | 0.050                |
| 9.5         | <5 × 10^-5     | <5 × 10^-5               | <5 × 10^-5    | 0.037                 | 0.02        | 0.800                |
| 11.7        | 0.0015         | <5 × 10^-5               | <5 × 10^-5    | 0.151                 | <5 × 10^-5  | 0.001                |

**Table 3. Demographic Information of the Validation of the Classification Tree**

| Sample | Healthy (n = 42) | HCC (n = 56) | P Value (α > .05) |
|--------|-----------------|--------------|------------------|
| Sex (Male/Female) | 28/14          | 38/18         | NS               |
| Age (yr) | 53 ± 11         | 55 ± 13       | NS               |
| Ethnicity (%) | NHW/AA/H/Asian 88/12/0/0 | 90/4/6/0 | NS               |
| Etiology (%) | HCV 56         | <.001         |
| HBV    | 3              | NS            |
| Alcohol | 10             | <.001         |
| Cryptogenic | 18             | .01           |
| Other  | 13             | NS            |
| MELD score | 5 ± 0.7       | 8 ± 2         | <.01             |
| ALT (IU/mL) | 21 ± 9      | 75 ± 40       | <.001            |
| AST (IU/mL) | 18 ± 5       | 111 ± 65      | .003             |
| Bilirubin (mg/dL) | 0.3 ± 0.2 | 1.5 ± 1       | .07              |
| AFP (ng/mL) | 1.8 ± 0.6     | 234,871 ± 3,592 | .003            |
| % <20 | 100           | 32            |
| % 20-200 | 0            | 45            |
| % >200 | 0             | 23            |
| TNM stage % (I/II/III/IV) | NA             | 13/24/38/25  |

NOTE. All data are presented as the mean ± SD.

**Table 4. Decision Tree Classification Results for Normal Samples Versus Different Sets of Liver Diseases**

| Condition | Sensitivity | Specificity |
|-----------|-------------|-------------|
| I.A. All non-cirrhosis liver disease vs. normal | 67% (25/38) | 66% (26/39) |
| I.B. HCV non-cirrhosis vs. normal | 74% (20/27) | 74% (29/39) |
| II.A. All cirrhosis vs. normal | 72% (26/36) | 72% (28/39) |
| II.B. HCV-cirrhosis vs. normal | 91% (20/22) | 91% (35/39) |
| III.A. All HCC vs. normal | 82% (47/57) | 90% (35/39) |
| III.B. HCV-HCC vs. normal | 89% (25/28) | 95% (38/39) |
| III.C. All HCV disease vs. normal | 79% (65/82) | 82% (32/39) |

NOTE. All subgroup A samples were the parent cohorts from which the HCV disease-associated cohorts (subgroup B) were selected. Subgroup C was the combination of HCV-non-cirrhosis and HCV-cirrhosis samples compared with normal samples. I indicates non-cirrhosis liver disease; II, cirrhosis; and III, HCC.
rithm analyses were applied, the Poon et al. study and our study demonstrate that SELDI protein profiling of serum can provide discriminating protein peaks in classifying chronic liver diseases and HCC.

In the emerging field of mass spectrometry–based protein profiling of body fluids, the profile itself is diagnostic, and extending its use does not depend on identification of the proteins in discriminating peaks. Of course, protein identification is still possible, and will add biological relevance to the findings. To move this type of analysis toward a clinical diagnostic application, it would be optimal to have a classification algorithm that has the ability to evaluate interspectral relationships between markers, and one with the capability of distinguishing multiple diagnoses simultaneously. The decision classification tree used herein is not effective for intraspectral analysis; its use was limited in that it is able to compare only two conditions when four sets required analysis. However, the methods described in this report illustrate the potential for developing a direct clinical assay, because the process involves minimal processing of serum, a fully automated loading and chip-binding procedure, and enough peak features to determine differences in disease states. This approach emphasizes peak/protein selection prior to classification and can allow the subsequent identification of potential biomarkers of known molecular mass. There is also enough flexibility in the process to allow inclusion of other clinical data from known tumor or disease markers (e.g., AFP, DCP, and GP73).

The development and testing of different classification algorithms for proteomic profiling data is continuing to evolve, and it remains to be determined if a single approach will be valid for all applications. Most likely it will be necessary to empirically evaluate multiple analysis algorithms for each disease state and determine how much processing of each spectra (baseline subtraction, instrument noise, and so forth) is necessary to attain maximal use of the features within the data. Previous applications of different genetic cluster analyses to unprocessed SELDI data have been questioned as to whether potential nonbiological features present in the spectra (<2,000 m/z) were useful in the classification process. In contrast, a separate report has shown that useful biological features were present in the 800 to 1,500 m/z range that were used in four separate models to accurately distinguish serum

| Table 5. Analysis of All 38 SELDI Peaks and Determined Serum Levels of 3 Marker Proteins (AFP, DCP, GP73) |
|---------------------------------------------------------------|
| **Condition** | **Sensitivity** | **Specificity** |
| Chronic HCV vs. HCV-HCC | 71% (20/28) | 64% (14/22) |
| Chronic HCV vs. HCV-HCC | 79% (22/28) | 86% (19/22) |
| HCV disease vs. HCV-HCC | 61% (17/28) | 78% (37/49) |
| HCV disease vs. HCV-HCC | 75% (21/28) | 92% (45/49) |

Abbreviations: Chronic HCV, non-cirrhosis HCV-infected samples; HCV disease, all non-cirrhosis and cirrhosis HCV-infected samples; HCV-HCC, HCV-infected HCC samples.
samples of ovarian cancer patients from healthy patients. Whatever methods ultimately evolve, we believe the algorithm should retain the flexibility to include known serum markers (such as AFP) in combination with the SELDI/matrix-assisted laser desorption/ionization peak profiles. This multiple marker approach has greater potential to improve the current single-marker analyses used in most cancer diagnostic assays.

What are the likely identities and functional properties of the different serum protein markers from our study and other serum profiling studies? Are these peaks only representative of acute-phase reactants, as has been a consistent criticism of the proteomic profiling of serum approach, or do the peaks reflect immune responses, viral infection responses, or cancer-specific proteins? Depending on the clinical characteristics associated with the sample, the peaks most likely represent components associated with each possibility. The clinical characteristics of the sample cohort that was analyzed herein (Table 1) and the differential protein profiling results that were obtained across the conditions illustrates this concept. Differential protein patterns were obtained across the three liver disease states, indicating the detection of more than just acute phase reactants. In previous studies, isolation of peptides bound to circulating serum albumin has indicated multiple fragments of proteins associated with cell growth and oncogenesis. In our serum-processing protocols, these same peptides would be stripped off albumin by the urea/CHAPS dilutions, which is one explanation for the presence and detection of the disease-specific peptides. Even if the lower-mass polypeptides represent fragments of acute phase proteins, these could be generated from proteases specific to the disease state, and thus the pattern of their appearance—and, ultimately, identification of the proteases that generated them—could provide direct insights into disease pathogenesis. Additionally, multiple tumor or disease-specific posttranslational modifications of serum proteins also have to be considered and evaluated as has been described for apolipoprotein AII. A mass spectrometry platform combined with an immune capture component could ultimately be the most effective means to assess these types of modifications of proteins found in complex clinical fluids.

We chose to use nonfractionation of the serum before protein chip analysis, because this allows a higher throughput and minimizes reproducibility problems for larger sets of serum. As proteomic tools specifically designed for serum fractionation and purifications evolve, a limited fractionation strategy or removal of major serum proteins such as albumin before analysis remain as viable options for future studies. Another possibility is to selectively enrich for major serum carrier proteins, because these could be the primary carriers of the low-mass peptides evaluated with our SELDI profiling strategy. A previous study examining the peptides bound to these serum carrier proteins indicated that discriminatory peptides previously observed in serum from ovarian cancer patients were enriched in signal intensity. Also, in our study, only one affinity surface, IMAC-Cu, was used. A combination of protein profiles derived from other affinity surfaces (e.g., hydrophobic, weak cationic, and strong anionic) could be combined with all of the discriminatory peaks found with the IMAC-Cu surface. Additionally, mass spectrometry platforms for evaluating proteins in clinical fluids will continue to evolve to provide improvements in dynamic mass ranges, increased resolution and peak detection sensitivities. As these different approaches are attempted, the results from our study can serve as a baseline for comparison to any future procedural improvements.

In conclusion, we present a phase 1 exploratory biomarker study that shows that serum protein profiling using SELDI-TOF can distinguish patients with HCC from those with chronic liver disease, particularly those with HCV infection. This exploratory study provides the basis for a larger phase 2 biomarker validation study currently being conducted by the National Cancer Institute’s Early Detection Research Network. Proteomic profiling of the serum from this cohort will allow further verification of the methods described herein, as well as provide a cohort for the evaluation of different mass spectrometry systems and other classification algorithms. Various purification and sequencing efforts to identify the low-mass protein biomarkers identified in this study are also ongoing. We anticipate that data from these studies may help to improve the outcome for patients with HCC by enabling the diagnosis to be made at an earlier stage of the disease when curative resection and/or transplantation treatment is possible.

References
1. El-Serag HB, Mason AC. Rising incidence of hepatocellular carcinoma in the United States. N Engl J Med 1999;340:745-750.
2. El-Serag HB, Mason AC. Key C. Trends in survival of patients with hepatocellular carcinoma between 1977 and 1996 in the United States. HEPATOLOGY 2001;33:62-65.
3. Bruis J, Llovet JM. Prognostic prediction and treatment strategy in hepatocellular carcinoma. HEPATOLOGY 2002;35:519-524.
4. Zaman SN, Melia WM, Johnson RD, Portmann BC, Johnson PJ, Williams R. Risk factors in development of hepatocellular carcinoma in cirrhosis: prospective study of 613 patients. Lancer 1985;1:1357-1360.
5. Lok AS, Lai CL. alpha-Fetoprotein monitoring in Chinese patients with chronic hepatitis B virus infection: role in the early detection of hepatocellular carcinoma. HEPATOLOGY 1989;9:110-115.
6. Sherman M, Peltzian KM, Lee C. Screening for hepatocellular carcinoma in chronic carriers of hepatitis B virus: incidence and prevalence of hepatocellular carcinoma in a North American urban population. HEPATOLOGY 1995;22:432-438.
7. Di Bisceglie AM, Hoofnagle JH. Elevations in serum alpha-fetoprotein levels in patients with chronic hepatitis B. Cancer 1989;64:2117-2120.
8. Colombo M, de Franchis R, Del Ninno E, Sangiovanni A, De Fazio C, Tommasini M, et al. Hepatocellular carcinoma in Italian patients with cirrhosis. N Engl J Med 1991;325:675-680.
9. Oka H, Tamori A, Kuroki T, Kobayashi K, Yamamoto S. Prospective study of alpha-fetoprotein in cirrhotic patients monitored for development of hepatocellular carcinoma. Hepatology 1994;19:61-66.
10. Paterson D, Garnett N, Trinchet JC, Auerousse MH, Mal F, Meicler C, et al. Prospective study of screening for hepatocellular carcinoma in Caucasian patients with cirrhosis. J Hepatol 1994;20:65-71.
11. Sangiovanni A, Del Ninno E, Fasani P, De Fazio C, Ronchi G, Romeo R, et al. Increased survival of cirrhotic patients with a hepatocellular carcinoma detected during surveillance. Gastroenterology 2004;126:1005-1014.
12. Zoli M, Magalotti D, Bianchi G, Gueli C, Marchesini G, Pisi E. Efficacy of a surveillance program for early detection of hepatocellular carcinoma. Cancer 1996;78:977-985.
13. Sheu JC, Sung JL, Chen DS, Lai MY, Wang TH, Yu JY, et al. Early detection of hepatocellular carcinoma by real-time ultrasonography. A prospective study. Cancer 1985;56:660-666.
14. Marrero JA, Sanna G, Rizzello P, Magalotti D, Bianchi G, Gueli C, et al. A prospective study of screening for hepatocellular carcinoma in black Americans. Hepatology 2003;37:1114-1121.
15. Aisaka H, Shimizu K, Miwa K. Immunohistochemical study of protein induced by vitamin K absence or antagonist II in hepatocellular carcinoma. J Surg Oncol 2003;84:89-93.
16. Nagaoka S, Yatsuhashi H, Hamada H, Yano K, Matsumoto T, Daikoku M, et al. The des-gamma-carboxy prothrombin index is a new prognostic indicator for hepatocellular carcinoma. Cancer 2003;98:2671-2677.
17. Kladney RD, Bulla GA, Guo L, Mason AL, Tollefson AE, Simon DJ, et al. GP73, a novel Golgi-localized protein upregulated by viral infection. Gene 2000;249:53-65.
18. Kladney RD, Cui X, Bulla GA, Brunt EM, Fimmel CJ, Expression of GP73, a resident Golgi membrane protein, in viral and nonviral liver disease. Hepatology 2002;35:1431-1440.
19. Komunyakaa MA, Mattu TS, Lovman MA, Evans AA, London WT, Semmes OJ, et al. Comparative proteomic analysis of de-N-glycosylated serum from hepatobiliary carriers reveals polypeptides that correlate with disease status. Proteomics 2004;4:826-838.
20. Poon TC, Yip TT, Chan AT, Yip C, Yip V, Mok TS, et al. Comprehensive proteomic profiling identifies serum proteomic signatures for detection of hepatocellular carcinoma and its subtypes. Clin Chem 2003;49:752-760.
21. Steel LF, Shumpett D, Trotter M, Seeholzer SH, Evans AA, London WT, et al. A strategy for the comparative analysis of serum proteomes for the discovery of biomarkers for hepatocellular carcinoma. Proteomics 2003;3:601-609.
22. Srinivas PR, Srivastava S, Hanash S, Wright GL Jr. Proteomics in early detection of cancer. Clin Chem 2001;47:1901-1911.
23. Adam BL, Vlahou A, Semmes OJ, Wright GL Jr. Proteomic approaches to biomarker discovery in prostate and bladder cancers. Proteomics 2001;1:1264-1270.
24. Wurtzbleher JD, Pawletz CP, Steeg PS, Petricoin EF 3rd, Liotta L. Proteomic approaches to the diagnosis, treatment, and monitoring of cancer. Adv Exp Med Biol 2003;532:59-68.
25. Adam BL, Qu Y, Davis JW, Ward MD, Clements MA, Cazares LH, et al. Serum protein fingerprinting coupled with a pattern-matching algorithm distinguishes prostate cancer from benign prostate hyperplasia and healthy men. Cancer Res 2002;62:3609-3614.
26. Cazares LH, Adam BL, Ward MD, Nasim S, Schellhammer PF, Semmes OJ, et al. Normal, benign, neoplastic, and malignant prostate cells have distinct protein expression profiles resolved by surface enhanced laser desorption/ionization mass spectrometry. Clin Cancer Res 2002;8:2541-2552.
27. Li J, Zhang Z, Rosenzweig J, Wang YY, Chan DW. Proteomics and bioinformatics approaches for identification of serum biomarkers to detect breast cancer. Clin Chem 2002;48:1296-1304.