Early Diagnostic Potential for Hepatocellular Carcinoma Using the SELDI ProteinChip System

Shuji Kanmura, Hirofumi Uto, Kazunori Kusumoto, Yoichi Ishida, Satoru Hasuike, Kenji Nagata, Katsuhiro Hayashi, Akio Ido, Sherri Oliver Stuver, and Hirohito Tsubouchi

Early detection of HCC increases the potential for curative treatment and improves survival. To facilitate early detection of HCC, this study sought to identify novel diagnostic markers of HCC using surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF/MS) ProteinChip technology. Serum samples were obtained from 153 patients with or without HCC, all of whom had been diagnosed with HCV-associated chronic liver disease. To identify proteins associated with HCC, serum samples were analyzed using SELDI-TOF/MS. We constructed an initial decision tree for the correct diagnosis of HCC using serum samples from patients with (n = 35) and without (n = 44) HCC. Six protein peaks were selected to construct a decision tree using this first group. The efficacy of the decision tree was then assessed using a second group of patients with (n = 29) and without (n = 33) HCC. The sensitivity and specificity of this decision tree for the diagnosis of HCC were 83% and 76%, respectively. For a third group, we analyzed sera from seven patients with HCC obtained before the diagnosis of HCC by ultrasonography (US) and from five patients free of HCC for the past 3 years. Use of these diagnostic markers predicted the diagnosis of HCC in six of these seven patients before HCC was clinically apparent without any false positives. Conclusion: Serum profiling using the SELDI ProteinChip system is useful for the early detection and prediction of HCC in patients with chronic HCV infection. (Hepatology 2007;45:948-956.)

Approximately 170 million people worldwide are infected with HCV, which when persistent can progress to HCC. The incidence of HCC is rising; in the United States over the past 2 decades, age-specific incidence has shifted toward younger people. IFN or combined IFN and ribavirin, which are currently the only effective treatments for chronic hepatitis C, reduce the occurrence of HCC. Some patients, however, do not receive IFN treatment or fail to clear HCV even with IFN treatment. In addition, a subset of individuals remain unaware that they are infected with HCV; in these patients, HCC may present only in the advanced stage. The prognosis of patients presenting with symptoms related to HCC is extremely poor. In contrast, early detection of HCC before the onset of clinical symptoms can lead to curative treatment, significantly improving prognosis.

Several methods developed for the diagnosis of HCC, including evaluation of serum markers, ultrasonography (US), computed tomography (CT), and magnetic resonance imaging, have been tested clinically. Alpha-fetoprotein (AFP) and des-gamma carboxy prothrombin (DCP), serum proteins that are elevated in HCC, have been the most widely used markers. Although routine screening offers the best chance for early tumor detection and improved survival, the reported sensitivities and specificities of elevated serum AFP and DCP levels vary significantly. In addition, AFP levels are elevated in only 30% to 40% of patients with HCC, particularly early in the disease process. Elevated AFP levels are also seen in patients with noncancerous conditions, such as cirrhosis.

Abbreviations: AFP, alpha-fetoprotein; AUC, area under the curve; CT, computed tomography; DCP, des-gamma carboxy prothrombin; m/z, mass-to-charge ratio; ROC, receiver operating characteristics; SELDI-TOF/MS, surface-enhanced laser desorption ionization time-of-flight mass spectrometry; US, ultrasonography.

From the 1Division of Gastroenterology and Hematology, Department of Internal Medicine, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan; 2Miyazaki Prefectural Industrial Support Foundation, Miyazaki, Japan; the 3Department of Experimental Therapeutics, Translational Research Center, Kyoto University Hospital, Kyoto, Japan; the 4Department of Epidemiology, Boston University School of Public Health, Boston, Massachusetts; the 5Department of Epidemiology, Harvard School of Public Health, Boston, Massachusetts; and the 6Digestive Disease and Life-style related Disease Health Research, Human and Environmental Sciences, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan.

Received June 29, 2006; accepted December 12, 2006.

This work was supported in-part by grants-in-aid from the Collaboration of Regional Entities for the Advancement of Technological Excellence (CREATE) from the Japan Science and Technology Agency, a grant (no. CA87982) from the United States National Institutes of Health, and a grant-in-aid (Research on Hepatitis and Biomedical Science, Japan) from the Ministry of Health, Labour and Welfare of Japan.

Address reprint requests to: Hirohito Tsubouchi, Digestive Disease and Life-style related Disease Health Research, Human and Environmental Sciences, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan. E-mail: htsubo@m2.kufm.kagoshima-u.ac.jp; fax: (81) 99-264-3504.

Copyright © 2007 by the American Association for the Study of Liver Diseases. Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/hep.21598

Potential conflict of interest: Nothing to report.
or exacerbations of chronic hepatitis, which confounds the screening results. Marrero et al.\textsuperscript{9} reported that DCP levels were more sensitive and specific than AFP testing for differentiating HCC from nonmalignant chronic liver disease. The usefulness of DCP for the detection of early HCC is limited, however. Wang et al.\textsuperscript{8} reported that the number of patients with small HCC (less than 2 cm) demonstrating elevations in DCP was low (56.5%). AFP-L3, the lectin lens culinaris agglutinin–bound fraction and one of the three AFP glycoforms, is the major glycoform of AFP elevated in the serum of HCC patients. At a cutoff level of 15% of total AFP, the reported sensitivities for differentiating HCC from nonmalignant liver disease are 75% to 96.9% with specificities of 90% to 92.0%.\textsuperscript{10,11}

AFP-L3 is closely related to poor differentiation and biologically malignant characteristics, such as portal vein invasion, of neoplastic cells,\textsuperscript{11,12} how useful this test is for the early detection of HCC is unclear. In addition, the diagnosis of small mass lesions using US or CT is relatively inaccurate. Thus, additional biochemical markers are necessary for specific detection of early HCC.

The development of proteomic array technology for serum profiling, in which a ProteinChip Array is coupled with surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF/MS; Ciphergen Biosystems Inc., Fremont, CA), has created a powerful tool for the discovery of new biomarkers. This technology has been successfully applied using samples from patients with prostate, ovarian, and gastric cancers. The great advantages of this method are speed, high-throughput capability, and the requirement of only a small amount of sample. Although serum AFP levels and US are the most common examination methods used for HCC surveillance, the classification tree algorithm detailed in this study provided a more accurate classification than these examination methods alone.\textsuperscript{13,14}

This study sought to assess and compare protein expression profiles of sera from patients with or without HCC on a background of chronic liver disease attributable to HCV infection. We assessed the ability of SELDI-TOF/MS ProteinChip technology to identify serum markers that could enable early HCC diagnosis.

**Patients and Methods**

**Samples.** The 153 male patients with chronic liver disease attributable to HCV infection were selected; serum samples were collected by the Faculty of Medicine of the University of Miyazaki (Miyazaki, Japan). All patients were negative for hepatitis B surface antigen. Seventy-seven of the patients were negative for HCC, which was confirmed by US or CT of the abdomen. Samples from 64 patients with HCC were obtained before treatment. Patients were randomly divided into two groups; the first analysis group was composed of 35 and 44 patients with and without HCC, respectively, whereas 29 and 33 patients with and without HCC, respectively, made up the second analysis group. The clinical characteristics of the first and second analysis groups were not significantly different except for the average age (Table 1). In conjunction with an ongoing cohort study, we also obtained prediagnostic sera from seven patients determined to have HCC within 1 year of US screening and five patients who have remained free of HCC for the past 3 years.\textsuperscript{15} These subjects constitute the third analysis group (Table 2). Twenty-six healthy volunteers without either liver neoplasia or HCV infection served as negative controls. After freezing and thawing once, all samples were separated into 20- to 30-μl aliquots and refrozen at –80°C until analysis.

**SELDI-TOF/MS.** For analysis, we used ProteinChip Arrays (CM10) with anionic surface chemistry. CM10 ProteinChip Arrays incorporate a carboxylate group that acts as a weak cation exchanger. Chips were rinsed with ultra-pure water and put into a bioprocessor (Ciphergen Biosystems, Inc.), a device that holds 12 chips and allows the application of larger volumes of serum to each chip array. Within the bioprocessor, the chips were washed twice with shaking on a platform shaker at a speed of 300 rpm for 5 minutes in 150 μl binding/washing buffer (50 mM sodium acetate, pH 4.5) per well. Five-microliter serum samples were denatured in 45 μl urea buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% dithiothreitol, and 2% ampholines), then diluted 1:9 in binding/washing buffer. After washing the chips extensively in binding/washing buffer, 100 μl of the denatured, diluted serum was applied to each chip spot. The bioprocessor was then sealed and shaken on a platform shaker for 40 minutes. Chips were then removed from the bioprocessor. After washing 3 times in binding/washing buffer, we rinsed the chips once in water. Each spot was then treated twice with 0.5 μl saturated sinapinic acid (SPA) (Nacalai Tesque Inc, Kyoto, Japan) and allowed to air-dry.

Arrays were analyzed using a ProteinChip Reader (ProteinChip Biology System II, Ciphergen Biosystems Inc.). Time-of-flight spectra were generated by laser shots collected in positive mode. Laser intensity ranged from 225 to 240, with a detector sensitivity of 6. An average of 65 laser shots per spectrum were performed. For mass accuracy calibration according to the manufacturer’s instructions, 500 nl of a mixture of mass standard calibration proteins (All-in-one Peptide Standard; Ciphergen Biosystems) were applied to single spot of the normal phase (NP20) chip array, followed by two applications of 1.0 μl
saturated SPA. The mass-to-charge ratio (m/z) of each the proteins captured on the array surface was determined according to externally calibrated standards.

Peak Detection, Data Analysis, and Decision Tree Classification. Peak detection was performed using Ciphergen ProteinChip Software, version 3.0.2 (Ciphergen Biosystems). Spectra between 1300 and 150,000 m/z were selected for analysis. Smaller masses were not analyzed, because these were determined to be artifacts of energy absorbing molecules. Spectra were normalized to total ion current intensity. In the preliminary examination, we observed significant noise in spectra with ranges less than 3000 m/z. In addition, no differences were apparent in the peaks of spectra at values greater than 10,500 m/z between 4 serum samples from patients with HCC and 4 samples from patients without HCC. Therefore, after baseline subtraction, we performed automatic peak detection in the optimized range of 3000 to 10,500 m/z, using peak auto-detection set to cluster, a first-pass signal/ noise ratio of 5, a minimal peak threshold of 20% for all spectra, and a cluster mass window of 0.3% mass.

Based on the peak intensities of the 55 signal clusters obtained, a decision tree was constructed from the first analysis group. For each sample, the intensity values for Table 1. Patient Characteristics in First and Second Analysis Groups

| 1st Analysis Group | Total | HCC§ | CLD§§ | P  |
|--------------------|-------|------|-------|----|
| Patients           | 79    | 35   | 44    |    |
| Age                | 66.7 ± 10.3**§§ | 72.7 ± 4.7 | 60.3 ± 13.3 | 0.007 |
| PLT* (X10^3/μL)    | 9.6 ± 5.3 | 11.5 ± 5.9 | 7.6 ± 4.2 | 0.001 |
| Albumin (g/dL)     | 3.6 ± 1.2 | 3.6 ± 0.5 | 3.5 ± 0.5 | NS |
| ALT** (IU/L)       | 56.6 ± 31.9 | 56.6 ± 25.5 | 56.7 ± 35.6 | NS |
| AFP*** (ng/mL)     | 209.4 ± 982.3 | 348 ± 1307 | 26 ± 25 | NS |
| DCP† (mAU/mL)      | 191.9 ± 538.5 | 299 ± 686 | 42 ± 43 | NS |
| HA†† (ng/mL)       | 353.6 ± 538.5 | 412 ± 480 | 293 ± 265 | NS |
| Diameter of the HCC (mm) | - | 23.1 ± 9.8 | - | |
| TMNstage††† (I/II/III/IV) | - | 18/14/3/0 | - | |

NOTE. Data are shown as the means±SD. Gender: male, statistical differences were determined by the Mann-Whitney U test. Values of p < 0.05 were considered to be statistically significant. §§§Although age differed between the 1st and 2nd analysis group, none of the other factors described were not different.

Abbreviation: *platelet count, **alanine aminotransferase, ***alpha fetoprotein, †des-γ-carboxy prothrombin, ††hyaluronic acid, †††TMN; primary tumor/lymph node/distant metastasis, §hepatocellular carcinoma, §§chronic liver disease.

Table 2. Patient Characteristics in the Third Analysis Groups

| 3rd Analysis Group | HCC††† Occurrence Within 1 Year | No HCC Occurrence Within 3 Years | P  |
|--------------------|--------------------------------|---------------------------------|----|
| Patients           | 7                              | 5                               |    |
| Age                | 72.8 ± 4.1                     | 75.8 ± 5.6                      | NS |
| PLT* (X10^3/μL)    | 14.8 ± 4.8                     | 8.1 ± 1.4                       | 0.04 |
| Albumin (g/dL)     | 4.1 ± 0.6                      | 4.0 ± 0.4                       | NS |
| ALT** (IU/L)       | 70.0 ± 44.9                    | 59.0 ± 34.3                     | NS |
| AFP*** (ng/mL)     | 195.2 ± 305.7                  | 23.4 ± 22.4                     | NS |
| DCP† (mAU/mL)      | 139.5 ± 226.6                  | 17.2 ± 4.6                      | 0.01 |
| HA†† (ng/mL)       | 310.6 ± 322.8                  | 459.6 ± 114.8                   | NS |

NOTE. Data are shown as the means±SD. Gender: male, statistical differences were determined by the Mann-Whitney U test. Values of p < 0.05 were considered to be statistically significant. NS indicates not significant.

Abbreviation: *platelet count, **alanine aminotransferase, ***alpha fetoprotein, †des-γ-carboxy prothrombin, ††hyaluronic acid, †††hepatocellular carcinoma.
each peak within the 3000-10,500 m/z range were input into Biomarker Patterns Software (Ciphergen Biosys-tems) and classified according to the tree analysis de-
dcribed.\textsuperscript{13,16} Decision trees classify spectrum patterns through sequential questioning, in which the next ques-
tion asked depends on the answer.\textsuperscript{17} With a decision tree, classification of patterns begins at the roof node, follow-
ing the appropriate links based on the answers obtained to
the questions posed at each node.

**Peak Reproducibility.** Reproducibility is critical for reliable disease diagnosis and early detection. We exam-
in ed the reproducibility of our assay system using pooled normal sera from 2 individuals.\textsuperscript{13} Four protein peaks ran-
domly selected over the course of the study were used to
calculate the coefficient of variance (CV) as described.\textsuperscript{18} We then determined the reproducibility of the SELDI
spectra, both within and between arrays (intra-assay and interassay, respectively). The intra-assay (spot-to-spot) CV was 10.2% for peak intensity and 0.25% for mass accuracy. The interassay (chip to chip) CV was 15.9% for peak intensity and 0.67% for mass accuracy. We also
observed minimal variation of day-to-day instrumenta-
tion (data not shown).

**Statistical Analysis.** Values shown are the means ±
SD. Statistical differences, including laboratory data and
individual peaks in SELDI-TOF/MS, were determined by the Mann-Whitney \textit{U} test. Values of \( P < 0.05 \) were
considered statistically significant. The discriminatory
power for each putative marker was described via receiver
operating characteristics (ROC) area under the curve
(AUC). These statistical analyses were performed using
STATVIEW 4.5 software (Abacus Concepts, Berkeley,
CA), SPSS software (SPSS Inc., Chicago, IL), or Cipher-
gen ProteinChip Software, version 3.0.2.

Sample numbers for the first group used to develop the
decision tree were small. A cross-validation approach us-
ing multiple decision trees would be more suitable for the
construction of a final decision tree model.\textsuperscript{19} In this study,
we validated the models using a 10-fold cross-validation
approach to construct the final decision tree model as
described previously.\textsuperscript{16,18} The result of the biomarker pat-
terns software using this approach differed from the clas-
sification and regression tree analysis by univariate
analysis (Mann-Whitney \textit{U} test).\textsuperscript{20}

**Results**

**Detection of HCC (Data Analysis).** We aimed to
identify a single peak protein or pattern of peaks that
could distinguish HCC patients from individuals without
HCC. Initially, we analyzed serum samples from the first
analysis group, a random 35 and 44 patients with and
without HCC, respectively, using the SELDI Protein-
Chip system. Peaks were detected automatically after
baseline subtraction using Ciphergen ProteinChip Soft-
ware, version 3.0.2.\textsuperscript{13} This analysis identified 55 signal
peak protein clusters, seen in the spectrum representa-
tions of the two groups (HCC and non-HCC) within the
3000 to 10,500 m/z range (Fig. 1). Eight protein peaks
were overexpressed, whereas 10 protein peaks were down-
regulated significantly in sera from HCC patients in com-
parison with those from patients without HCC. The
mean amplitudes of the peaks for the 2 patient groups are
shown in Table 3.

**Structure of the Decision Tree.** Decision trees are
flowchart-like tree structures that repeatedly split data sets
into subsets in accordance with the given cancer versus
nontumor classification task. Each classifier, a simple rule
applied to each patient, queries only one mass. Serum
samples isolated from 35 HCC patients and 44 chronic
liver disease patients without HCC served as the training
set. Using the normalized peak intensities of these 55
signal clusters, we constructed and evaluated decision
trees using the training set. Peaks with a high discrimina-
tory power were used to create 6 mass classifiers (m/z =
3444, 3890, 4067, 4435, 4470, and 7770) of differing
complexities. Although 2 of these classifiers did not differ
significantly between patients with and without HCC
(m/z = 3444 and 3890), the decision tree generated using
the combination of these 6 protein peaks correctly classi-
fied 97% of HCC samples (Fig. 2, Table 3).

**Testing the Decision Tree.** To determine the accu-
racy and validity of the algorithm, we reevaluated the
decision tree (Fig. 2) that had been constructed using the
training set, using the first test set (second analysis group).
To evaluate the classification performance, we deter-
mined the sensitivity and specificity of the algorithm for
the differentiation between patients with and without
HCC. The decision tree algorithm correctly diagnosed
83% (24 of 29) patients with HCC and 76% (25 of 33)
patients without HCC. Although the ROC AUC of each
of the 6 mass classifiers were 0.70, 0.61, 0.71, 0.64, 0.66,
and 0.70, which individually were more discriminatory
than existing serum marker methods, the decision tree
algorithm had highest discriminatory power (Tables 3, 4).
Twenty-six healthy volunteers were all correctly identified
as free of HCC. The accuracy of the algorithm for HCC
diagnosis was higher than that of other known tumor
markers (Table 4).

**Decision Tree Predicts HCC Occurrence.** The most
fundamental requirement for serum-based marker detec-
tion is identification of carcinoma at an early stage when
treatment has the greatest impact on prognosis. We inves-
tigated the specificity of our classification system using a
second test set (3rd analysis group) of samples taken from 7 patients 1 year before the development of HCC and 5 patients with chronic liver disease remaining free of HCC for at least 3 years. Six of the 7 (86%) patients who later developed HCC were classified to the HCC group using the classifiers described previously (Fig. 2, Table 3), even though the HCC was undetectable by US at the time of serum testing. All 5 patients without HCC were classified to the non-HCC group. These results indicate that this decision tree analysis is useful for the early diagnosis of HCC.

Discussion

Proteomic analyses of sera and liver tissues from patients with HCC associated with HBV or HCV infection has been used to identify new biomarkers predicting HCC development, leading to improved prognosis.21-27 Because many analyses use 2-dimensional electrophoresis, the proteins used in such investigations must typically be greater than 10,000 daltons in molecular weight.21,25-29 Analyzing serum or another body fluid that is easy to obtain from patients to predict disease or evaluate treatment efficacy would be ideal. In this study, we used the SELDI ProteinChip system to analyze serum samples from patients with HCC. This affinity-based mass spectrometric method, which combines chromatography and MS, is suitable for the analysis of both proteins and low-molecular-weight peptides.14 Although we did not identify a single effective biomarker, we developed a new decision tree, using a cross-validation approach, that uses a multimarker algorithm of 6 proteins capable of diagnosing and predicting HCC at least 1 year before the appearance of clinically detectable disease in patients infected with HCV.

Ninety percent of the protein content of serum is composed of 10 proteins, including albumin and IgG; an additional 12 proteins make up 90% of the remaining 10%. Thus, only 1% of the protein content of serum is of interest as potential biomarkers in proteomic studies.30 Several proteomic methods combine high-resolution separation of complex protein mixtures with additional protein identification methods, such as MS. To identify the low abundance proteins of interest, one must remove the most abundant proteins from the serum by techniques such as immunodepletion. These methods are only reliable if the assumption that biomarkers are not bound to major circulating proteins is correct. If bound to these proteins, low-abundance biomarkers would be lost by im-

Fig. 1. Analysis of sera from patients with and without HCC (spectrum). Serum samples were applied to CM10 ProteinChip Arrays. Representative spectra from patients in each of the 2 groups (HCC and non-HCC) are presented. The horizontal axis indicates protein mass to charge (m/z), whereas the longitudinal axis designates the relative intensity. Lower highlight panels represent the peaks used in the classifier described in Fig. 2. Peaks of (A) 3444, (B) 3890, (C) 4067, (D) 4435, (E) 4470, and (E) 7770 m/z are shown.
m/z HCC (n = 35) Non-HCC (n = 44) p value

Overexpressed proteins
4067† 3.94 ± 4.56 1.92 ± 1.79 0.03
4470† 8.36 ± 4.28 6.49 ± 3.99 0.01
6433 13.61 ± 10.10 8.94 ± 8.42 0.02
6632 26.87 ± 18.11 18.20 ± 15.09 0.02
7770† 8.40 ± 5.94 5.26 ± 4.42 0.0002
8138 12.76 ± 14.78 5.86 ± 5.37 0.006
8605 4.39 ± 3.08 3.20 ± 2.45 0.02
8934 16.10 ± 10.69 10.36 ± 7.26 0.009

Downregulated proteins
3326 1.27 ± 0.74 2.10 ± 1.21 0.003
3398 0.90 ± 0.77 2.43 ± 2.50 0.0008
3444† 2.02 ± 1.18 2.45 ± 1.50 0.2
3816 1.98 ± 1.17 3.45 ± 2.84 0.002
3826 1.65 ± 4.95 2.51 ± 3.53 0.002
3890† 3.12 ± 1.35 3.31 ± 1.41 0.2
4135 3.45 ± 2.24 5.08 ± 3.86 0.01
4175 5.49 ± 9.46 12.32 ± 14.63 0.001
4435† 1.23 ± 1.73 2.31 ± 2.63 0.006
4658 1.14 ± 0.80 1.94 ± 1.71 0.007
4791 2.42 ± 1.33 4.04 ± 3.27 0.004
6979 0.82 ± 0.52 1.19 ± 0.67 0.01

NOTE. Data are shown as the means ± SD, statistical differences were determined using the Mann-Whitney U test, †Peaks selected in final classification model by decision tree analysis.

Abbreviation: *hepatocellular carcinoma.
HCC. Recently, Schwegler et al.\textsuperscript{16} reported an algorithm using the seven peaks that scored highest by SELDI TOF/MS. The determined classification tree, however, could not distinguish HCC from chronic liver disease; using 38 SELDI peaks, the sensitivity and specificity (61% and 76%) for distinguishing chronic HCV from HCV-HCC were lower than those determined for the decision tree constructed in this study. Schwegler et al. demonstrated that their sensitivity and specificity values increased to 75% and 92%, respectively, when AFP/DCP/GP73 was added to their classification model. In our model, although the sensitivity and specificity values increased to 75% and 92%, respectively, when AFP/DCP/GP73 was added to their classification model. In our model, although the sensitivity increased to 92%, specificity did not increase (52%) after the addition of AFP/AFP-L3/DCP to our classification. Serum GP73 levels, which were not available for examination in our study, or other as-yet-unknown characterizations of these patients may affect the predictive capability of this method. Although the sensitivity and specificity (92% and 90%) of another proteomics study using SELDI to distinguish chronic liver disease from HCC were higher than those determined in our study, greater than 63% of the study population examined exhibited advanced HCC (stage III and IV).\textsuperscript{16,36} Only 14% of the HCC patients included in our study population had stage III or IV disease (Table 1), which likely accounts for the differences in the peaks used in the two studies. The characteristics of the patients with HCC will likely affect both the sensitivity and specificity significantly. Thus, our decision tree is more suitable for the diagnosis of early HCC than any previously reported methods.\textsuperscript{16,36}

Although serum AFP level greater than 400 ng/ml serves as a useful method for the diagnosis of HCC,\textsuperscript{37} this detection method is insufficiently sensitive to detect small HCCs.\textsuperscript{38} Although the utility of several other markers has been shown to be superior to AFP in detecting early HCC,\textsuperscript{22,39,40} these markers were determined in patients with clinically apparent HCC. Thus, the sensitivity/specificity also may not be sufficient to detect early HCC. Our classification tree was able to predict cancer occurrence before HCC was clinically apparent by US. In the third analysis group, we correctly predicted the progression of 86% of the patients to HCC from their prediagnostic

**Table 4. Comparisons of Hepatocellular Carcinoma Diagnostic Rates for the Multiple Marker and Three Additional Tumor Marker Analyses in the Second Analysis Group**

| Markers                  | Sensitivity | Specificity | ROC AUC**** |
|-------------------------|-------------|-------------|-------------|
| Multiple-marker         | 83% (24/29) | 76% (25/33) | 0.79        |
| AFP* (>20 ng/mL)        | 41% (12/29) | 67% (22/33) | 0.57        |
| AFP-L3** (>15%)         | 17% (5/29)  | 88% (29/33) | 0.56        |
| DCP†,*** (>40 mAU/mL)   | 39% (11/28) | 81% (26/32) | 0.64        |

NOTE. †excluding subject whose data could not be obtained.  
Abbreviation: *alpha fetoprotein, **Lens culinaris agglutinin-reactive fraction of alpha-fetoprotein, *** des-γ-carboxy prothrombin, ****receiver operating characteristic area under the curve.
serum samples. To screen high-risk patients with chronic liver disease, such as that associated with HCV infection, our multi-marker analysis could help distinguish those patients for which the combined examination of US, CT, and arterial portography would be recommended.

In their investigation of differential protein expression in HBV-associated and HCV-associated HCC, Kim et al. identified 60 proteins displaying significant changes in expression levels between nontumorous and tumorous tissues. Forty-six of these proteins demonstrated an association with viral infection. We analyzed the sera of patients with HBV-associated HCC; the expression of a number of protein markers differed between HCV and HBV infections (data not shown). The biological and pathogenic activities of these 2 viruses are different; the molecular mechanisms underlying the development of hepatitis and hepatocarcinogenesis also may differ between HBV and HCV infections. Our analysis of the proteome using the SELDI technique demonstrates that this method also may be useful for investigation of the molecular mechanisms of hepatocarcinogenesis on the background of different viral infections.

A number of the peaks may represent doubly charged peaks; for example, the peak at 4067 m/z may be the doubly charged form of the 8138-m/z peak. One of the peaks in Table 3 included in the classification model also may be a doubly charged peak (3890/7770 m/z), which could affect the independent variables. To clarify this possibility, one must identify the individual proteins. The major limitation of the SELDI technique is that identification of individual proteins is often complicated. Lee et al. however, recently isolated complement C3a as a candidate biomarker in human chronic hepatitis C and HCV-related HCC using the SELDI-TOF MS system after serum fractionation, 2-dimensional gel electrophoresis, in-gel digestion, and MS. We are now identifying the single protein represented by the 8138-m/z peak; 3 candidate proteins are known. Although we have to confirm these results by western blotting, the peak at 4067 m/z does not appear to be the doubly charged peak of the 8138-m/z peak by SELDI immunoassay. Although the serum levels of no single protein are sufficient to detect early HCC from the results of ROC AUC, identification of proteins altered in the disease may help analyze the molecular mechanisms underlying HCC development and may help identify new therapeutic targets or modalities for the treatment or prevention of HCC.

In patients with HCV infection, serum profiling using the SELDI ProteinChip system is useful both for the early detection of HCC and to distinguish HCC from chronic liver disease in the absence of HCC. Our ability to identify proteomic alterations in serum samples from HCC patients suggests that the SELDI ProteinChip system may be useful to identify proteins associated with HCC in the hopes of developing new therapeutic targets.

Acknowledgments: We thank Hiroyuki Nakao for suggestions concerning statistical analyses. The authors thank Yuko Nakamura and Yuka Takahama for their technical assistance.

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. Yoshida H, Shiratori Y, Moriyama M, Arakawa Y, Ide T, Sata M, et al. Interferon therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. IHIT Study Group. Inhibition of Hepatocarcinogenesis by Interferon Therapy. Ann Intern Med 1999;131:174-181.
3. Heathcote EJ. Prevention of hepatitis C virus-related hepatocellular carcinoma. Gastroenterology 2004;127:S294–302.
4. 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.
5. Ishi M, Gama H, Chida N, Ueno Y, Shinzawa H, Takagi T, et al. Simultaneous measurements of serum alpha-fetoprotein and protein induced by vitamin K absence for detecting hepatocellular carcinoma. South Tohoku District Study Group. Am J Gastroenterol 2000;95:1036-1040.
6. Okuda H, Nakamichi T, Takatsu K, Saito A, Hayashi N, Takasaki K, et al. Serum levels of des-gamma-carboxy prothrombin measured using the revised enzyme immunoassay kit with increased sensitivity in relation to clinicopathologic features of solitary hepatocellular carcinoma. Cancer 2000;88:544-549.
7. Grazi GL, Mazziotto A, Legnani C, Jovine E, Miniero R, Gallucci A, et al. The role of tumor markers in the diagnosis of hepatocellular carcinoma, with special reference to the des-gamma-carboxy prothrombin. Liver Transpl Surg 1995;1:249-255.
8. Wang CS, Lin CL, Lee HC, Chen KY, Chiang MF, Chen HS, et al. Usefulness of serum des-gamma-carboxy prothrombin in detection of hepatocellular carcinoma. World J Gastroenterol 2005;11:6115-6119.
9. Marrero JA, Su GL, Wei W, Emmick D, Conjeevaram HS, Fontana RJ, et al. Des-gamma carboxyprothrombin can differentiate hepatocellular carcinoma from nonmalignant chronic liver disease in american patients. Hepatology 2003;37:1114-1121.
10. Takekta K, Okada S, Wain N, Hlaing NK, Wind KM. Evaluation of tumor markers for the detection of hepatocellular carcinoma in Yangon General Hospital, Myanmar. Acta Med Okayama 2002;56:317-320.
11. Khien VV, Mao HV, Chinh TT, Ha PT, Bang MH, Lac BV, et al. Clinical evaluation of lentil lectin-reactive alpha-fetoprotein-L3 in histology-proven hepatocellular carcinoma. Int J Biol Markers 2001;16:105-111.
12. Oka H, Saito A, Ito K, Kumada T, Satomura S, Kasugi H, et al. Multi-center prospective analysis of newly diagnosed hepatocellular carcinoma with respect to the percentage of Lens culinaris agglutinin-reactive alpha-fetoprotein. J Gastroenterol Hepatol 2001;16:1378-1383.
13. Adam BL, Qu Y, Davis JW, Ward MD, Clements MA, Cazes 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.
14. Paradis V, Degos F, Dargere D, Pham N, Belghiti J, Degott C, et al. Identification of a new marker of hepatocellular carcinoma by serum protein profiling of patients with chronic liver diseases. Hepatology 2005;41:40-47.
15. Uto H, Hayashi K, Kusumoto K, Hasuieke S, Nagata K, Kodama M, et al. Spontaneous elimination of hepatitis C virus RNA in individuals with persistent infection in a hyperendemic area of Japan. Hepatol Res 2006;34:28-34.
16. Schwegler EE, Cazares L, Steel LF, Adam BL, Johnson DA, Semmes OJ, et al. SELDI-TOF MS profiling of serum for detection of the progression of chronic hepatitis C to hepatocellular carcinoma. HEPATOLOGY 2005;41:634-642.

17. Duda RO, Hart PE, Stork DG. Pattern classification. 2nd ed. Hoboken, NJ: Wiley-Interscience, 2001.

18. Scarlett CJ, Saxby AJ, Nielsen A, Bell C, Samra JS, Hugh T, et al. Proteomic profiling of cholangiocarcinoma: diagnostic potential of SELDI-TOF MS in malignant bile duct stricture. HEPATOLOGY 2006;44:658-666.

19. Ambroise C, McLachlan GJ. Selection bias in gene extraction on the basis of microarray gene-expression data. Proc Natl Acad Sci USA 2002;99:6562-6566.

20. Lim SO, Park SJ, Kim W, Park SG, Kim HJ, Kim YI, et al. Proteome analysis of HCC proteome analysis of hepatocellular carcinoma. Biochem Biophys Res Commun 2002;291:1031-1037.

21. Capurro M, Wanless IR, Sherman M, Deboer G, Shi W, Miyoshi E, et al. Glypican-3: A novel serum and histochemical marker for hepatocellular carcinoma. Gastroenterology 2003;125:89-97.

22. Hippo Y, Watanabe K, Watanabe A, Midorikawa Y, Yamamoto S, Ihara S, et al. Identification of soluble NH2-terminal fragment of Glypican-3 as a serological marker for early-stage hepatocellular carcinoma. Cancer Res 2004;64:2418-2423.

23. Yoon SK, Lim NK, Ha SA, Park YG, Choi JY, Chung KW, et al. The human cervical cancer oncogene protein is a biomarker for human hepatocellular carcinoma. Cancer Res 2004;64:5434-5441.

24. Yokoyama Y, Kuramitsu Y, Takashima M, Iizuka N, Toda T, Terai S, et al. Proteomic profiling of proteins decreased in hepatocellular carcinoma from patients infected with hepatitis C virus. Proteomics 2004;4:2111-2116.

25. Kim W, Oe Lim S, Kim JS, Ryu YH, Byeon JY, Kim HJ, et al. Comparison of proteome between hepatitis B virus- and hepatitis C virus-associated hepatocellular carcinoma. Clin Cancer Res 2003;9:5493-5500.

26. Steel LF, Shumpert 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.

27. Steel LF, Mattu TS, Mehta A, Hebestreit H, Dwek R, Evans AA, et al. A proteomic approach for the discovery of early detection markers. DisMarkers 2001;17:179-189.

28. Liang CR, Leow CK, Neo JC, Tan GS, Lo SL, Lim JW, et al. Proteome analysis of human hepatocellular carcinoma tissues by two-dimensional difference gel electrophoresis and mass spectrometry. Proteomics 2005;5:2258-2271.

29. Tirumalai RS, Chan KC, Prieto DA, Issaq HJ, Comrads TP, Veenstra TD. Characterization of the low molecular weight human serum proteome. Mol Cell Proteomics 2003;2:1096-1103.

30. Chignard N, Beretta L. Proteomics for hepatocellular carcinoma marker discovery. Gastroenterology 2004;127:s120-s125.

31. Villanueva J, Martorella AJ, Lawlor K, Philip J, Fleisher M, Robbins RJ, et al. Serum peptide patterns that distinguish metastatic thyroid carcinoma from cancer-free controls are unbiased by gender and age. Mol Cell Proteomics 2005;6:1840-1852.

32. Scarlett CJ, Smith RC, Saxby A, Nielsen A, Samra JS, Wilson SR, et al. Proteomic classification of pancreatic adenocarcinoma tissue using protein chip technology. Gastroenterology 2006;130:1670-1678.

33. Won Y, Song HJ, Kang TW, Kim JJ, Han BD, Lee SW. Pattern analysis of serum proteome distinguishes renal cell carcinoma from other urologic diseases and healthy persons. Proteomics 2003;3:2310-2316.

34. Zhu XD, Zhang WH, Li CL, Xu Y, Liang WJ, Tien P. New serum biomarkers for detection of HBV-induced liver cirrhosis using SELDI protein chip technology. World J Gastroenterol 2004;10:2327-2329.

35. 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.

36. Sokolski M, Magliarisi C, Campagna P, Leto G, Bonfissuto G, Riili A, et al. Usefulness of alpha-fetoprotein in the diagnosis of hepatocellular carcinoma. Anticancer Res 2003;23:1747-1753.

37. Sherman M, Alphafetoprotein: an obituary. J Hepatol 2001;34:603-605.

38. Song BC, Chung YH, Kim JA, Choi WB, Suh DD, Yoon JH, et al. Transforming growth factor-beta1 as a useful serologic marker of small hepatocellular carcinoma. Cancer 2002;94:175-180.

39. Miura N, Maeda Y, Kanbe T, Yazama H, Takeda Y, Sato R, et al. Serum human telomerase reverse transcriptase messenger RNA as a novel tumor marker for hepatocellular carcinoma. Clin Cancer Res 2005;11:3205-3209.

40. Honda M, Kaneko S, Kawai H, Shirota Y, Kobayashi K. Differential gene expression between chronic hepatitis B and C hepatic lesion. Gastroenterology 2001;120:955-966.

41. Lee IN, Chen CH, Sheu JC, Lee HS, Huang GT, Chen DS, et al. Identification of complement C3a as a candidate biomarker in chronic hepatitis C and HCV-related hepatocellular carcinoma using a proteomics approach. Proteomics 2006;6:2865-2873.