Hepatocellular carcinoma (HCC) is an increasingly prevalent clinical problem worldwide and is the third most common cause of cancer-related death. Cirrhosis of any etiology is the most common risk factor for HCC development. Over 90% of HCCs develop on a cirrhotic liver resulting from either chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infections, alcohol abuse, or accumulation of fat referred as nonalcoholic steatohepatitis. Patients at risk for developing HCC should be entered into surveillance programs. Alpha-fetoprotein (AFP) is widely used as a surveillance and detection test for HCC among patients with cirrhosis, despite its limited performance, particularly in early-stage HCC. Apart from AFP, other markers (e.g., lectin-bound AFP [AFP-L3], des-gamma carboxyprothrombin [DCP], and glypican-3) have been proposed for HCC detection. However, recent studies showed that neither DCP nor AFP-L3 presented better performance characteristics than AFP for the diagnosis of early-stage HCC, and that neither DCP nor AFP is optimal to complement ultrasound in the detection of early HCC. Development of novel biomarkers for the early detection of HCC thus remains an important target before a breakthrough appears on HCC surveillance and early intervention. The aim of this study was to identify, using a proteomic approach, a biomarker that could improve AFP performance as a surveillance test for HCC among patients with cirrhosis.
Patients and Methods

Study Design and Patient Characteristics. Plasma samples were collected following informed consent from patients enrolled at the University of Michigan (Ann Arbor, MI) (Cohort 1) and from patients enrolled at the Cancer Control Unit of the National Cancer Institute of Thailand, Bangkok (Cohort 2). Assays were performed at the Fred Hutchinson Cancer Research Center (Seattle, WA). The study was performed in compliance with and after approval from the respective institutional review boards of all sites. At the University of Michigan, HCC was diagnosed according to the American Association for the Study of Liver Diseases (AASLD) guidelines. Early-stage HCC was defined as Barcelona Clinic Liver Cancer staging system (BCLC) stage A, according to AASLD guidelines, and cirrhosis was defined as previously described. At the National Cancer Institute (NCI) of Thailand, HCC diagnosis was based on a clinical algorithm, including imaging (i.e., ultrasonography [US] and computerized tomography) and biochemistry (i.e., AFP and liver-function enzyme testing). A previous study on patients from the NCI of Bangkok has shown that this diagnosis algorithm is over 95% specific against histopathology for detection of HCC in this context. A total of 312 patients, including 131 patients with HCC and 96 with cirrhosis, were selected from the two cohorts for this study. The characteristics of these patients are shown in Supporting Table 1.

Plasma Proteomic Profiling. Plasma was immuno-depleted of human albumin, transferrin, immunoglobulin, antitrypsin, and haptoglobin using the Multiple Affinity Removal Column (Agilent Technologies, Santa Clara, CA), and proteins from the immuno-depleted fractions were separated using the Alliance 2-D Bioseparations System (Waters Corporation, Milford, MA). The resulting protein fractions were further separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Gels were stained with colloidal Coomassie blue G-250, and each lane was cut into pieces. Gel pieces were destained and digested by trypsin to generate peptide solution. The generated peptide samples were analyzed by liquid chromatography/electrospray ionization/tandem mass spectrometry (MS/MS), equipped with a two-dimensional (2D) nano-HPLC (high-performance liquid chromatography) device (Eksigent, Dublin, CA) coupled to a hybrid LTQ-OrbiTrap (Thermo Scientific, Waltham, MA) mass spectrometer. The raw data file was converted to the m/z XML generic format and searched against the human International Protein Index protein sequence database using the X!Tandem Search Engine. To obtain reliable protein identifications from the search results, PeptideProphet and ProteinProphet, statistical tools that compute peptide and protein probabilities based on peptides assigned to MS/MS spectra, were used. Protein abundance was calculated as a function of the total number of spectra assigned to the protein.

Enzyme-Linked Immunosorbent Assay. Plasma levels of osteopontin (OPN) were measured using a commercial enzyme-linked immunosorbent assay kit (R&D Systems, Inc., Minneapolis, MN) and 50 μL of diluted (1:100) plasma samples. Samples were added to the plates precoated with the OPN antibody first and incubated for 2 hours at room temperature. After four washes, 200 μL of the OPN antibody conjugated to horseradish peroxidase were added and incubated for 2 hours at room temperature. After four washes, 200 μL of tetramethylbenzidine substrate solution were added and incubated for 30 minutes in the dark, followed by the addition of 50 μL of stop solution. Absorbance was measured at 450 nm with wavelength correction at 540 nm, using a SpectraMax Plus384 spectrophotometer (Molecular Devices, Sunnyvale, CA).

Statistical Analysis. To summarize test performance on the whole range of thresholds, receiver operating characteristic (ROC) curves were plotted for each biomarker test. Area under the ROC curve (AUC) was calculated, and its 95% confidence interval (CI) was calculated via 1,000 bootstrap samples. We calculated the optimal cutoffs using the maximum sum of sensitivity and specificity as well as using the minimum distance to the top-left corner of the ROC curve. The complementary property of OPN to AFP for the
diagnosis of HCC was illustrated by comparing ROC curves using the “or” rule of a logistic regression with OPN and AFP in the model to that with only AFP in the model (i.e., predictor is coded as 1 if either AFP or OPN is above its respective prespecified threshold). All analyses were repeated after stratifying the samples for viral etiology, AFP levels, and HCC stage. Sensitivity and specificity and their 95% CIs were calculated for AFP and OPN. Differences of sensitivities and specificities for differentiating HCC and cirrhotic controls between OPN and AFP and their 95% CIs were calculated using 1,000 bootstrap samples.

Results

**MS Profiling Identified Higher Plasma Levels of OPN in HCC Patients, Compared to Patients With Cirrhosis.** Extensive MS analysis after a multidimensional protein separation strategy composed of 2D HPLC, followed by SDS-PAGE, was applied to plasma samples collected from 17 patients with HCC and 18 with liver cirrhosis. To reduce variability, all selected patients were male, with AFP values below 20 ng/mL. OPN was identified by MS with high confidence, based on the assignment of 12 unique peptides resulting in 40.4% coverage of the sequence (Fig. 1A). Though OPN was detected in the majority (81%) of the samples analyzed, the number of peptide hits assigned to OPN was significantly higher in HCC plasma (mean = 22.12), compared to cirrhosis plasma (mean = 2.5) (P = 0.03), indicative of higher OPN levels in the plasma of patients with HCC (Fig. 1B).

**Circulating OPN Levels in HCC Patients From Cohort 1.** We first analyzed OPN levels in a cohort (Cohort 1) established in the United States and including a majority of HCC and cirrhosis patients with CHC (55% and 59%, respectively) and a majority (60%) of early-stage HCC (Table 1). OPN levels were measured blinded to clinical data in a total of 173 plasma samples collected from 40 HCC patients, 73 cirrhosis patients, 32 with CHC, and 28 healthy controls. Plasma levels of OPN were significantly higher in HCC plasma (mean = 271.4 ng/mL) than in cirrhosis (mean = 86.2 ng/mL; P < 0.0001), CHC (mean = 52.6 ng/mL; P < 0.0001), and healthy controls (mean = 32 ng/mL; P < 0.0001) (Fig. 2).

**Performance of OPN in Cohort 1.** We further evaluated the performance of OPN in discriminating HCC and cirrhosis patients in Cohort 1. The AUC for OPN (0.76; 95% CI: 0.66-0.85) was higher than for AFP (0.71; 95% CI: 0.60-0.82) (Fig. 3A). The combination of OPN and AFP further increased the AUC (0.82; 95% CI: 0.73-0.91). When only HCV-associated HCCs were compared with HCV-associated cirrhosis, the AUC for AFP decreased (0.64; 95% CI: 0.49-0.80), whereas OPN had an even higher AUC (0.80; 95% CI: 0.69-0.91) (Fig. 3B). For HCV patients, the addition of AFP to OPN only marginally increased the AUC for OPN alone (0.81; 95% CI: 0.7-0.93). When only early-stage HCCs, defined in this study as BCLC stage A, were compared with cirrhosis, OPN had, again, a higher AUC (0.73; 95% CI: 0.62-0.85) than AFP (0.68; 95% CI: 0.54-0.82) (Fig. 3C). The combination of OPN and AFP further increased the AUC (0.81; 95% CI: 0.70-0.91). Finally, when only HCC with AFP levels below 20 ng/mL were included, OPN AUC remained high (0.75; 95% CI: 0.64-0.87), whereas, as expected, AFP AUC was low (0.59; 95% CI: 0.43-0.76) (Fig. 3D).

The performance of OPN in these patient groups is further described in Table 1. When using the currently recommended clinical cutoff for AFP (20 ng/mL) and the best cutoff for OPN determined using the minimal distance to the top-left corner in the ROC curve (91 ng/mL), OPN had a better performance than AFP, with a sensitivity of 74% (95% CI: 60-88), compared
to 53% (95% CI: 37-69) for AFP. When AFP and OPN were combined in a logistic regression model, sensitivity increased to 85% (95% CI: 74-96) and specificity was 63% (95% CI: 52-74). At an optimal combination of AFP at 20 ng/mL and of OPN at 149 ng/mL, the combination of OPN and AFP had a sensitivity of 71% (95% CI: 57-86) and a specificity of 86% (95% CI: 79-94). For HCV patients, the performance of OPN further increased (sensitivity, 82%; 95% CI: 65-79), whereas AFP performance decreased (sensitivity, 46%; 95% CI: 26-66), when compared to all HCCs. For early-stage HCC, OPN had, again, a better performance than AFP, with a sensitivity of 75%, compared to 46% for AFP. When AFP and OPN were combined for early-stage HCC, sensitivity increased to 83% (95% CI: 69-98). At an optimal OPN cut-off level of 149 ng/mL, the combination of OPN and AFP had a sensitivity of 71% (95% CI: 53-89) and a specificity of 86% (95% CI: 78-94) for early-stage HCC.

To further validate the performance described above, we performed a 10-fold cross-validation analysis for the combination AFP and OPN (Supporting Table 2). The average from 10 runs showed similar sensitivity and specificity averages on classifier calibration and classifier validation.

**Circulating OPN Levels in HCC Patients From Cohort 2.** We subsequently analyzed OPN levels in an independent cohort (Cohort 2) established in Thailand and including a majority (59%) of HCC with CHB and a majority of advanced HCC (Table 1). OPN levels were measured blinded to clinical data in a total of 139 plasma samples collected from 91 HCC patients, 23 with cirrhosis or CHB, and 25 healthy controls. Plasma levels of OPN were significantly higher in HCC patients, compared with all control groups. OPN plasma levels were significantly higher in HCC (mean = 565.8 ng/mL; P < 0.0001) than in cirrhosis and CHB (89.2 ng/mL; P < 0.0001) and also greater than in healthy controls (47.7 ng/mL; P < 0.0001) (Fig. 4A).

**Performance of OPN in Cohort 2.** ROC analysis was performed to evaluate the performance of OPN in distinguishing HCC patients from patients with cirrhosis and chronic HBV. Overall, AFP performance
was greater in this cohort, compared to Cohort 1, as expected, because elevated levels of AFP was part of the clinical algorithm for the detection of HCC. However, even with these diagnosis criteria and as observed for Cohort 1, the AUC for OPN (0.93; 95% CI: 0.88-0.98) was higher than for AFP (0.86; 95% CI: 0.80-0.92) (Fig. 4B). The combination of AFP and OPN had an even higher AUC than OPN alone (0.96; 95% CI: 0.92-0.99). When only HBV-associated HCC patients were included, OPN AUC was also higher (0.97; 95% CI: 0.94-1.00) than AFP AUC (0.90; 95% CI: 0.84-0.97) (Fig. 4C). Finally, when only HCC patients with AFP levels below 20 ng/mL were included, OPN AUC remained high (0.87; 95% CI: 0.75-0.99) (Fig. 4D).

The performance of OPN in these patient groups is further described in Table 2. When using the currently recommended clinical cutoff for AFP (20 ng/mL) and an OPN cutoff of 91 ng/mL (determined based on Cohort 1 results), OPN had a better performance than AFP, with a sensitivity of 93% (95% CI: 88-98), compared to 78% (95% CI: 70-86) for AFP. The best cutoff for OPN, determined using the minimal distance to the top-left corner in the ROC curve, was 156 ng/mL in this cohort. When AFP at 20 ng/mL and OPN at 156 ng/mL were combined, sensitivity was 95% (95% CI: 90-99) and specificity was 96% (95% CI: 87-100). For HBV patients, the performance of OPN alone was particularly high, with 95% sensitivity. At the OPN cut-off level of 156 ng/mL, the combination of OPN and AFP had a sensitivity of 96% and a specificity of 100%.

**OPN Levels in Prediagnostic Samples.** In a pilot study, we investigated whether OPN levels would be elevated in samples collected before HCC diagnosis in patients enrolled in Cohort 1. At the time of the study, a total of 22 cirrhosis patients with prospectively collected blood specimens developed HCC during follow-up. Among these 22 patients, 19 were diagnosed with early- or very-early-stage HCC and 3 were diagnosed with intermediate-stage HCC (Supporting Table 3). At the time of diagnosis, only 8 of these 22 patients had AFP levels above 20 ng/mL, whereas 19 of them had OPN levels above 91 ng/mL, the optimal cutoff determined by analysis of Cohort 1 samples (Fig. 5A). Samples collected 6-12 months before diagnosis were available for 12 of these 22 patients; among them, 2 patients had AFP levels above 20 ng/mL, whereas 10 had OPN levels above 91 ng/mL (Fig.
Samples collected 12-24 months before diagnosis were available for 10 patients; among them, 1 had AFP levels above 20 ng/mL and 6 had OPN levels above 91 ng/mL (Fig. 5C). Altogether, 87% of these patients had OPN levels above the cutoff within 2 years preceding HCC diagnosis. OPN values were below 91 ng/mL in all samples collected more than 24 months before diagnosis (Fig. 5D). A similar analysis was performed on 22 cirrhosis patients who did not develop HCC during follow-up (data not shown). Median values of OPN in these patients were 54 ng/mL at time 0, 60.5 ng/mL 6-12 months before, 54 ng/mL 12-24 months before, and 60 ng/mL 24-48 months before, demonstrating the steady levels of OPN over the years in these patients, in contrast to the progressive increase of OPN levels in patients who developed HCC during follow-up. Among these 22 cirrhotic patients who did not develop HCC, 1 patient had OPN values above 91 ng/mL in all 7 samples collected over a period of 4 years. Another patient had OPN values above 91 ng/mL at time 0 and 12 months before, whereas all additional 4 samples collected 12-53 months before had OPN values below 91 ng/mL. It has to be noted that AFP was also above 20 ng/mL in this patient at time 0.

Discussion

This study aimed at identifying novel biomarkers for HCC surveillance in patients with cirrhosis and represents a first demonstration of the utility of using plasma proteomic profiling to identify novel biomarkers of early HCC. Comparative proteomic profiling of plasma obtained from patients with HCC or cirrhosis identified higher levels of circulating OPN in HCC. OPN is a secreted phosphoprotein that binds alphaV-integrins and cluster of differentiation (CD)44 families of receptors. Elevated expression of OPN has been associated with tumor invasion, progression, or metastasis in multiple cancers, and OPN has been proposed as a promising target for cancer therapy. In HCC, an elevated plasma level of OPN is regarded as a potential prognostic biomarker, and overexpression of OPN is closely correlated with intrahepatic metastasis,
early recurrence, and a worse prognosis.\textsuperscript{17-20} This study is, therefore, the first demonstrating a potential utility of OPN in early HCC detection. The utility of OPN in early HCC detection may be relevant to recent reports suggesting that beside its important role in metastasis, OPN expression is also critical for tumor growth of human HCC,\textsuperscript{21} and that down-regulation of OPN suppresses growth of HCC via induction of apoptosis.\textsuperscript{22}

Overall, OPN sensitivity was better than AFP in differentiating HCC cases from cirrhosis controls in both cohorts included in this study and in all HCC subgroups tested. The best performance was, however, obtained by combining OPN and AFP. The combination of both markers enhanced sensitivity and specificity in detecting HCC, indicating that these two markers are complementary. An important goal in cancer surveillance is the detection of preclinical tumors.

Table 2. Sensitivity and Specificity of AFP, OPN, and AFP+OPN in Cohort 2 Using AFP Clinical and OPN Optimal Cut-off Values

<table>
<thead>
<tr>
<th>Cohort 2</th>
<th>Cutoffs (ng/mL)</th>
<th>Sensitivity (%) (95% CI)</th>
<th>Specificity (%) (95% CI)</th>
</tr>
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<tbody>
<tr>
<td>Cirrhosis/CHB versus HCC</td>
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<td></td>
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<tr>
<td>AFP</td>
<td>20</td>
<td>78 (70-86)</td>
<td>96 (87-100)</td>
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<tr>
<td>OPN</td>
<td>91</td>
<td>93 (88-98)</td>
<td>61 (41-81)</td>
</tr>
<tr>
<td>OPN</td>
<td>156</td>
<td>82 (75-90)</td>
<td>96 (87-100)</td>
</tr>
<tr>
<td>AFP+OPN</td>
<td>(\text{AFP} = 20) or (\text{OPN} = 91)</td>
<td>97 (93-100)</td>
<td>56 (36-77)</td>
</tr>
<tr>
<td>AFP+OPN</td>
<td>(\text{AFP} = 20) or (\text{OPN} = 156)</td>
<td>95 (90-99)</td>
<td>96 (87-100)</td>
</tr>
<tr>
<td>HBV-cirrhosis/CHB versus HBV-HCC</td>
<td>20</td>
<td>84 (74-94)</td>
<td>94 (82-100)</td>
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<tr>
<td>OPN</td>
<td>91</td>
<td>95 (88-100)</td>
<td>69 (46-92)</td>
</tr>
<tr>
<td>OPN</td>
<td>156</td>
<td>85 (76-95)</td>
<td>100 (--)</td>
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<tr>
<td>AFP+OPN</td>
<td>(\text{AFP} = 20) or (\text{OPN} = 91)</td>
<td>98 (95-100)</td>
<td>62 (39-86)</td>
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<tr>
<td>AFP+OPN</td>
<td>(\text{AFP} = 20) or (\text{OPN} = 156)</td>
<td>96 (92-100)</td>
<td>100 (--)</td>
</tr>
<tr>
<td>Cirrhosis/CHB versus HCC (AFP &lt;20 ng/mL)</td>
<td>91</td>
<td>85 (69-100)</td>
<td>100 (--)</td>
</tr>
<tr>
<td>OPN</td>
<td>156</td>
<td>74 (55-93)</td>
<td>96 (88-100)</td>
</tr>
</tbody>
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AFP, alpha-fetoprotein; OPN, osteopontin; 95% CI, 95% confidence interval; CHB, chronic hepatitis B; HCC, hepatocellular carcinoma; HBV, hepatitis B virus.

Fig. 5. Plasma levels of OPN in prediagnosis samples. OPN was measured in plasma collected from 22 patients (A) at time of diagnosis, (B) 6-12 months before diagnosis, (C) 12-24 months before diagnosis, and (D) more than 24 months before diagnosis. Graphs also show levels of AFP in the same samples. The dotted lines represent the cutoffs for AFP (20 ng/mL) and for OPN (91 ng/mL).
In a pilot study, we showed that OPN values remained intact for up to 2 years preceding HCC diagnosis. This study is an important first step in the evaluation of OPN as a marker of early-stage HCC and represents a phase I/II study, as defined by the guidelines established by the NCI, for the development of biomarkers for the early detection of cancer. A phase I/II study consists of case-control studies to evaluate the performance of a new marker, in this case OPN, to diagnose early-stage cancer. As controls, we selected patients with cirrhosis recommended for surveillance, which is the design suggested by the NCI’s guidelines for biomarker development. Such phase I or II studies are critical to appropriately design prospective studies for the early detection of HCC. Overall, we showed that OPN is a marker of early-stage HCC and complements AFP in a small validation study. In addition, the performance of OPN was evaluated in two distinct patient populations with different disease etiologies. This study had, however, some limitations, such as the small sample size in the patient subgroups and the fact that the patients in Cohort 2 were, in part, diagnosed using AFP, therefore creating a bias in favor of AFP performance in this cohort. This study was also performed in plasma collected using a standardized protocol. Further analysis will be needed to determine factors in sample preparation and analysis that might affect the performance of OPN. The next step is a large-scale validation in a phase II study that will also include the analysis of OPN against other currently used markers, such AFP-L3 and DCP, and integration of long-term outcome data. This will be followed by a phase III prospective study aimed at determining whether OPN alone or in combination with AFP complements US as a screening test to find lesions that have a high likelihood of cure or whether OPN is a useful marker for risk stratification.

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References