Real-time PCR allelic discrimination assays were designed using TaqMan single nucleotide polymorphism (SNP) genotyping assays (Applied Biosystems, Foster City, CA, USA). Typing reagents for the *HFE* gene SNPs G845A (dbSNP ID: rs1800562; TaqMan SNP genotyping assay ID, C_1085595_10), which confers a C282Y mutation, and C187G (dbSNP ID: rs1799945; TaqMan SNP genotyping assay ID, C_10856009_10), which confers a H63D mutation, were purchased from Applied Biosystems. Genotyping of the A193T SNP (dbSNP ID: rs28934888) in the *HFE* gene, which confers a S65C mutation, was performed using the following primers and probes: primer F (GACCAGCTGTTCGTGTTCTATGAT), primer R (CCACATCTGGCTTGAAATTCTACTG), probe F (ACGGCGACTCTCAT, labeled with the dye VIC), and probe R (CGGCGACACTCAT, labeled with the dye FAM), with a custom TaqMan genomic assay. Briefly, 5 ng of DNA were mixed with the Allelic Discrimination Assay Mix (900 nM of each forward and reverse primer and 200 nM of each reporter dye (FAM or VIC)-labeled probe) and TaqMan Universal PCR Master Mix (Applied Biosystems). The PCR conditions were 50°C for 2 min with AmpErase uracil *N*-glycosylase and 95°C for 10 min, followed by 40 cycles of 92°C for 15 s, and 60°C for 1 min. Genotypes were assessed by the TaqMan allele-specific assay method using the ABI Prism 7000 Sequence Detection System, according to the manufacturer's protocols (Applied Biosystems). All genotypes were scored using the allelic discrimination program of ABI software.

Follow-up of subjects

At the beginning of 2001, ALT measurements were obtained an average of twice a year until September 2005. An increase in ALT levels to greater than 35 IU/l was considered an ALT flare-up. For those subjects in whom all prior ALT measurements had been normal, the follow-up period for ALT flare-up spanned from 2001 until (1) the date of the initial ALT flare-up, (2) the last sequential ALT measurement, or (3) the conclusion of the study in September 2005, whichever occurred first.

Statistical analysis

All statistical analyses were performed using STATVIEW 4.5 software (Abacus Concepts, Berkeley, CA, USA) or SPSS software (SPSS, Chicago, IL, USA). The cumulative incidence of ALT flare-up was analyzed by the Kaplan-Meier method; the differences in the curves were evaluated by a log-rank test. Multivariate analysis was performed by Cox proportional hazards models. Fisher's exact test or the Mann-Whitney *U* test was also used, where appropriate. A *P* value of less than 0.05 was considered statistically significant.

Results

In 2000, 159 of the 440 (35.7%) HCV carriers were considered to have PNALT. Of the 159 subjects with PNALT, 58 subjects did not have ALT measurements beginning in 2001 and were excluded from the present analyses. Table 1 shows the characteristics of the remaining 101 subjects with PNALT who were included in this study to analyze the incidence of ALT flare-up.

The mean follow-up period was 2.8 years in the 101 subjects with PNALT. Over this 2.8-year period, 21 subjects experienced an ALT flare-up, with an estimated five-year cumulative flare-up incidence of 31.8%, determined using the Kaplan-Meier method (Fig. 1). The cumulative incidence of ALT flare-up after 2001 was similar between men and women (data not shown).

Based on univariate analysis, age in 2000, HCVcAg level in 1995, sex, and HCV serotype were not associated with an increased rate of ALT flare-up in subjects with PNALT (Table 2). An ALT level of 20–34 IU/l [hazard ratio (HR) = 4.72] and a serum ferritin level ≥90 ng/ml (HR = 2.96) in the most recently available data up to 2000 were associated with a significantly increased rate of ALT flare-up. In addition, although

Table 1. Demographic and virologic data for 101 subjects positive for HCV core antigen or HCV RNA that had at least four annual ALT measurements available between 1993 and 2000

Characteristics	*n* = 101
Age[a]	71.4 ± 7.9 (101)
Sex, male / female	22 / 79
Body mass index	22.2 ± 2.8 (101)
Alcohol intake (none / occasional or daily)	68 / 33
HCV core antigen (pg/ml)[b]	194.8 ± 196.4 (90)
HCV serotype (I/II)[c]	57 / 34
AST (IU/l)[d]	28.2 ± 7.2 (101)
ALT (IU/l)[d]	19.6 ± 5.0 (101)
γ-GTP (IU/l)[d]	16.6 ± 9.5 (101)
HbA1c (%)[d]	5.6 ± 0.5 (78)
Total cholesterol (mg/dl)[d]	179 ± 35.1 (101)
Triglyceride (mg/dl)[d]	122.4 ± 74.3 (101)
Hemoglobin (g/dl)[d]	13.2 ± 1.0 (79)
Serum ferritin (ng/ml)[d]	94.6 ± 54.7 (88)

Data are shown as means ± SD (number of subjects examined)
HCV, hepatitis C virus; ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GTP, γ-glutamyl transpeptidase; HbA1c, hemoglobin A1c
[a] In 2000
[b] Excluding subjects with HCV core antigen levels below 8 pg/ml
[c] Excluding subjects whose HCV serotype was undetermined.
[d] Most recently available data between 1996 and 2000

Table 2. Results of univariate analysis for ALT flare-up in subjects with persistently normal ALT

Variable	Number of patients	Hazard ratio	95% CI	*P* value
Age (years)[a]				
<65	15	1.0		
≥65	86	0.63	0.21–1.87	0.40
Sex				
Female	79	1.0		
Male	22	1.19	0.40–3.55	0.76
Body mass index				
<25	82	1.0		
≥25	19	1.36	0.526–3.51	0.53
Alcohol intake				
none	68	1.0		
occasional or daily	33	1.10	0.45–2.70	0.83
HCVcAg[b] (pg/ml)				
<100	46	1.0		
≥100	55	2.34	0.91–6.04	0.71
HCV serotype[c]				
Type 1	57	1.0		
Type 2	34	1.18	0.49–2.85	0.08
AST (IU/l)[d]				
<30	60	1.0		
≥30	41	1.70	0.72–4.00	0.23
ALT (IU/l)[d]				
<20	51	1.00		
20–34	50	4.72	1.59–14.03	<0.01
γ-GTP (IU/l)[d]				
<20	76	1.0		
≥20	25	1.08	0.42–2.80	0.87
HbA1c (%)[d]				
<5.9	51	1.0		
≥5.9	27	1.22	0.44–3.44	0.70
Total cholesterol (mg/dl)[d]				
<180	54	1.0		
≥180	47	1.16	0.49–2.73	0.74
Triglyceride (mg/dl)[d]				
<120	66	1.0		
≥120	35	1.90	0.81–4.48	0.14
Hemoglobin (g/dl)[d]				
<14	61	1.0		
≥14	18	1.88	0.71–4.96	0.20
Serum ferritin (ng/ml)[d]				
<90	45	1.0		
≥90	43	2.96	1.17–7.49	0.02
HFE H63D				
HH (wild)	87	1.0		
HD (mutation)	7	3.52	1.18–10.49	0.02

CI, confidence interval
[a] In 2000
[b] HCV core antigen
[c] Excluding subjects whose HCV serotype was undetermined
[d] Most recently available data between 1996 and 2000.

none of the subjects carried the C282Y or S65C *HFE* mutation or were homozygous for 63D/D in the *HFE* gene, we observed an association between the H63D *HFE* mutation and ALT flare-up in PNALT subjects (HR = 3.52). In a multivariate regression analysis including ALT, serum ferritin level, and presence of the *HFE* H63D mutation as variables, ALT (HR = 5.59), serum ferritin levels (HR = 3.10), and *HFE* H63D mutation (HR = 4.75) remained significant independent factors associated with the incidence of ALT flare-up in sub-

Table 3. Results of multivariate analysis for ALT flare-up in subjects with persistently normal ALT

Variable		Hazard Ratio (95% CI)	*P* value
ALT[a]	20–34 IU/l	5.59 (1.78–17.55)	0.003
Serum ferritin[a]	≥90 ng/ml	3.10 (1.21–8.01)	0.019
HFE H63D	Mutation, HD	4.75 (1.51–14.90)	0.008

[a]Most recently available data between 1996 and 2000

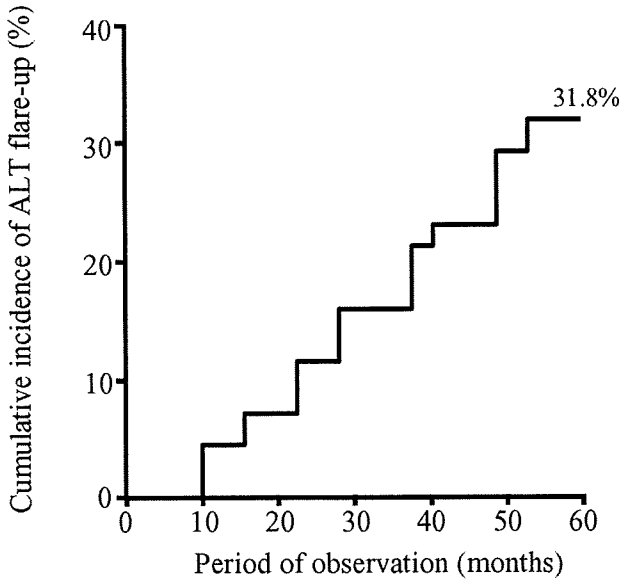

Fig. 1. Cumulative incidence of alanine aminotransferase (*ALT*) flare-up in subjects with persistently normal ALT levels, based on the Kaplan-Meier method

jects with PNALT (Table 3). In addition, serum levels of ferritin were significantly higher in subjects with the H63D mutation than in subjects without the mutation (153.8 ± 73.3 ng/ml in subjects with the 63HD genotype vs. 89.4 ± 51.3 ng/ml in subjects with the 63HH genotype, $P = 0.043$). The incidence of ALT flare-up was also significantly higher in subjects with the 63HD genotype than those with the 63HH genotype (57.1% vs. 19.5%, $P = 0.042$).

Discussion

This study examined a HCV hyperendemic area, where the prevalence of anti-HCV antibodies and persistent HCV infection are 20.6% and 70.7%, respectively.[22,24] In the present analysis, we focused on ALT flare-up in subjects with PNALT, and demonstrated that subjects with PNALT were at risk for ALT flare-up. Specifically, basal ALT levels and serum ferritin levels before ALT-flare-up and an *HFE* mutation (H63D) were correlated with ALT flare-up in PNALT subjects.

Previously, there has not been a clear definition of PNALT, either in terms of normal ALT levels or the time period of observation.[11,25–28] One report defined PNALT as three consecutive measurements within the normal ALT range during a 6-month period.[11] Puoti et al.[16] suggested that HCV carriers with normal ALT levels need to be observed for at least 18 months before they can be categorized as PNALT patients. Recently, Alberti[29] reported that the prevalence of cases with significant fibrosis was higher in studies in which PNALT status was determined from shorter observation periods or fewer ALT measurements than in studies in which PNALT classification was based on a longer observation period or more ALT tests. For this reason, we chose to define PNALT as having ALT levels within the normal range (<35 IU/l) over at least four independent, annual measurements. In addition, the PNALT guideline recommends that patients with ALT levels greater than 30 IU/l be treated the same as patients with chronic hepatitis C in Japan. We also analyzed the association between ALT flare-up and other factors if normal ALT levels were defined as values less than 30 IU/l. Eighty-five subjects with low PNALT (all ALT values ≤ 30 IU/l) were included to analyze the incidence of ALT flare-up (ALT > 30 IU/l). In a multivariate regression analysis including ALT levels (20–30 IU/l), serum ferritin levels, and the presence of the *HFE* H63D mutation as variables, ALT [HR = 3.02; 95% confidence interval (CI), 1.25–7.30] remained an independent factor associated with the incidence of ALT flare-up in subjects with PNALT (all ALT levels ≤ 30 IU/l). These results suggest that ALT levels are the most important factor for ALT flare-up in our study.

Okanoue et al.[30] previously showed that ALT levels increased in 86% of PNALT patients over 5 years. In our study, the estimated 5-year cumulative flare-up incidence was 31.8%, lower than the incidence rate reported by Okanoue et al. Only 33 of 80 (45%) PNALT patients without flare-up were followed until September 2005, and the mean follow-up period without flare-up was 2.9 years. It is possible that the ALT flare-up rate is lower in our study owing to the short follow-up period or the small number of subjects.

Our study suggests that ALT flare-up may be associated with higher serum ferritin levels, as previously reported.[31] Serum ferritin levels correlate with iron loading, a factor linked to HCV-associated fibrosis progression.[32] Serum iron, serum ferritin, and transferrin saturation are commonly elevated in patients with HCV.[33,34] The levels of these serum iron markers are indicators of hepatic iron stores, which may affect hepatic stellate cell activation and fibrosis progression.[32,35] Despite the links between HCV infection and hepatic iron load, the association between serum iron levels and ALT flare-up in patients with PNALT is not well understood. Vendemiale et al.[31] reported that an impaired redox state confers an increased risk of ALT flare-up in HCV carriers with PNALT (ALT levels ≤ 40 IU/l, measured every 2 months for at least 6 months); thus, an altered hepatic oxidative balance may have prognostic significance with respect to disease activity.[31] Serum ferritin levels correlate with hepatic iron. Excess hepatic iron storage may induce an altered hepatic redox state,[31,36] with high levels of intrahepatic iron accelerating liver injury and ALT flare-up. Thus, the storage of intrahepatic iron may contribute to liver injury, leading to fibrosis and HCC. Recently, Furutani et al.[37] reported that iron overload induces mitochondrial injury and increases the risk of HCC development in transgenic mice expressing the HCV polyprotein. They also showed that HCV transgenic mice fed an excess-iron diet showed significantly higher ALT levels in their serum than did control mice fed the same excess-iron diet (at 6 months after initiation of feeding). These results indicate that a combination of persistent HCV infection and iron overload may influence ALT flare-up in subjects with PNALT.

The presence of heterozygous *HFE* mutations is associated with higher hepatic iron stores and advanced fibrosis stage in patients with chronic hepatitis C.[33,38] Although heterozygous *HFE* mutations are rare in our study population, there was a significant association between an H63D mutation in the *HFE* gene and ALT flare-up in subjects with PNALT. We also observed that HCV carriers with PNALT who had the H63D *HFE* mutation had higher serum ferritin levels. Although individuals heterozygous for this *HFE* mutation are at low risk of iron overload,[39] this mutation in HCV carriers with PNALT may slowly affect hepatic iron levels and contribute to ALT flare-up over an extended period of time.

ALT levels in HCV-infected patients can be influenced by other factors, such as alcohol consumption or serum HCV RNA.[40–43] Although histological examinations were not performed, no association was observed between either alcohol consumption or HCVcAg levels, used as a correlate of HCV RNA, and ALT flare-up in this study. In addition, there was no correlation between baseline ALT levels and ferritin levels in subjects with PNALT (data not shown). We focused on HCV carriers with PNALT only, and the small number of subjects may be one reason for these results.

Although Shiffman et al.[44] reported that currently no parameters can be used to identify patients at elevated risk for progressive liver disease, the present results suggest that screening for serum ferritin levels and the H63D *HFE* mutation may help identify PNALT subjects at higher risk for ALT flare-up. Furthermore, to make appropriate decisions for interferon therapy, it is important to clarify whether ALT status correlates with liver disease progression. Although it remains unclear whether liver cirrhosis or HCC occurs in individuals with PNALT, recently, both Tanaka et al.[6] and our group[20] reported that elevated serum ALT levels prior to HCC diagnosis were positively associated with an increased risk of HCC. We found that subjects with at least four repeatedly elevated ALT measurements were at increased risk for HCC compared to patients with PNALT.[20] Subjects with fluctuating ALT levels experienced an age- and sex-adjusted HCC rate threefold that of subjects with normal ALT, although this association was not statistically significant. These results suggest that ALT elevation and, possibly, ALT flare-up are associated with an increased risk of HCC. Therefore, HCV carriers with PNALT exhibiting high serum ferritin levels and *HFE* H63D mutation should be considered candidates for antiviral therapy. Okanoue et al.[30] also have recommended antiviral treatment for HCV carriers with PNALT, depending on the results of follow-up blood chemistry and liver histology. We suggest that serum ferritin levels and H63D mutation in the *HFE* gene should also be examined for these patients.

In summary, we identified a high prevalence of HCV carriers with PNALT within a HCV hyperendemic community in Japan. Although HCV carriers are at relatively low risk for HCC, they can experience ALT flare-up, which is associated with an increased incidence of HCC. This study suggests that a subset of HCV carriers exhibit normal, consistently stable ALT levels and do not require liver biopsy or treatment. However, HCV carriers with PNALT whose serum ALT levels are near the upper limit of a normal range and whose serum ferritin levels are higher than 90 ng/ml or who carry the H63D *HFE* mutation are at increased risk for ALT flare-up. Because increases in serum ALT are associated with an elevated risk for HCC, these PNALT HCV patients should be considered for antiviral treatment and liver biopsy.

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Early Diagnostic Potential for Hepatocellular Carcinoma Using the SELDI ProteinChip System

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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.[1] IFN or combined IFN and ribavirin, which are currently the only effective treatments for chronic hepatitis C, reduce the occurrence of HCC.[2,3] 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.[4-9] In addition, AFP levels are elevated in only 30% to 40% of patients with HCC, particularly early in the disease process.[6] 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.

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or exacerbations of chronic hepatitis, which confounds the screening results. Marrero et al.[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.[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 of AFP-L3 as a method of detecting HCC range from 75% to 96.9% with specificities of 90% to 92.0%.[10,11] Because the high percentage of AFP-L3 observed in HCC is closely related to poor differentiation and biologically malignant characteristics, such as portal vein invasion, of neoplastic cells,[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.[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.[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% ampholites), 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

Table 1. Patient Characteristics in First and Second Analysis Groups

1st Analysis Group	Total	HCC§	CLD§§	P
Patients	79	35	44	
Age	66.7 ± 10.9§§§	72.7 ± 4.7	60.3 ± 13.3	0.007
PLT* (X10⁴/uL)	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	-	
2nd Analysis Group	**Total**	**HCC§**	**CLD§§**	**P**
Patients	62	29	33	
Age	72.2 ± 8.4§§§	73.6 ± 5.9	71.1 ± 10.2	NS
PLT* (X10⁴/UL)	10.0 ± 5.2	13.3 ± 5.8	7.6 ± 2.9	0.002
Albumin (g/dL)	4.0 ± 0.7	3.9 ± 1.0	4.0 ± 0.6	NS
ALT** (IU/L)	57.8 ± 33.6	57.2 ± 27.1	58.5 ± 38.7	NS
AFP*** (ng/mL)	61.5 ± 155.8	89.9 ± 189.9	19.2 ± 20.6	NS
DCP† (mAU/mL)	100.5 ± 271.4	163.4 ± 396.6	31.2 ± 26.2	NS
HA†† (ng/mL)	461.6 ± 522.6	286.7 ± 224.1	561.6 ± 615.2	NS
Diameter of the HCC (mm)	-	25.1 ± 10.5	-	
TMNstage††† (I/II/III/IV)	-	15/8/5/1	-	

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 statistical significant. NS indicates not 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.

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 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* (X10⁴/uL)	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 statistical significant.

Abbreviation: NS, not significant.

*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 Biosystems) and classified according to the tree analysis described.[13,16] Decision trees classify spectrum patterns through sequential questioning, in which the next question asked depends on the answer.[17] With a decision tree, classification of patterns begins at the roof node, following 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 examined the reproducibility of our assay system using pooled normal sera from 2 individuals.[13] Four protein peaks randomly selected over the course of the study were used to calculate the coefficient of variance (CV) as described.[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 instrumentation (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 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 Ciphergen ProteinChip Software, version 3.0.2.

Sample numbers for the first group used to develop the decision tree were small. A cross-validation approach using multiple decision trees would be more suitable for the construction of a final decision tree model.[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.[16,18] The result of the biomarker patterns software using this approach differed from the classification and regression tree analysis by univariate analysis (Mann-Whitney U test).[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 ProteinChip system. Peaks were detected automatically after baseline subtraction using Ciphergen ProteinChip Software, version 3.0.2.[13] This analysis identified 55 signal peak protein clusters, seen in the spectrum representations 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 downregulated significantly in sera from HCC patients in comparison 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 noncancer 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 discriminatory 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 classified 97% of HCC samples (Fig. 2, Table 3).

Testing the Decision Tree. To determine the accuracy 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 determined 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 detection is identification of carcinoma at an early stage when treatment has the greatest impact on prognosis. We investigated the specificity of our classification system using a

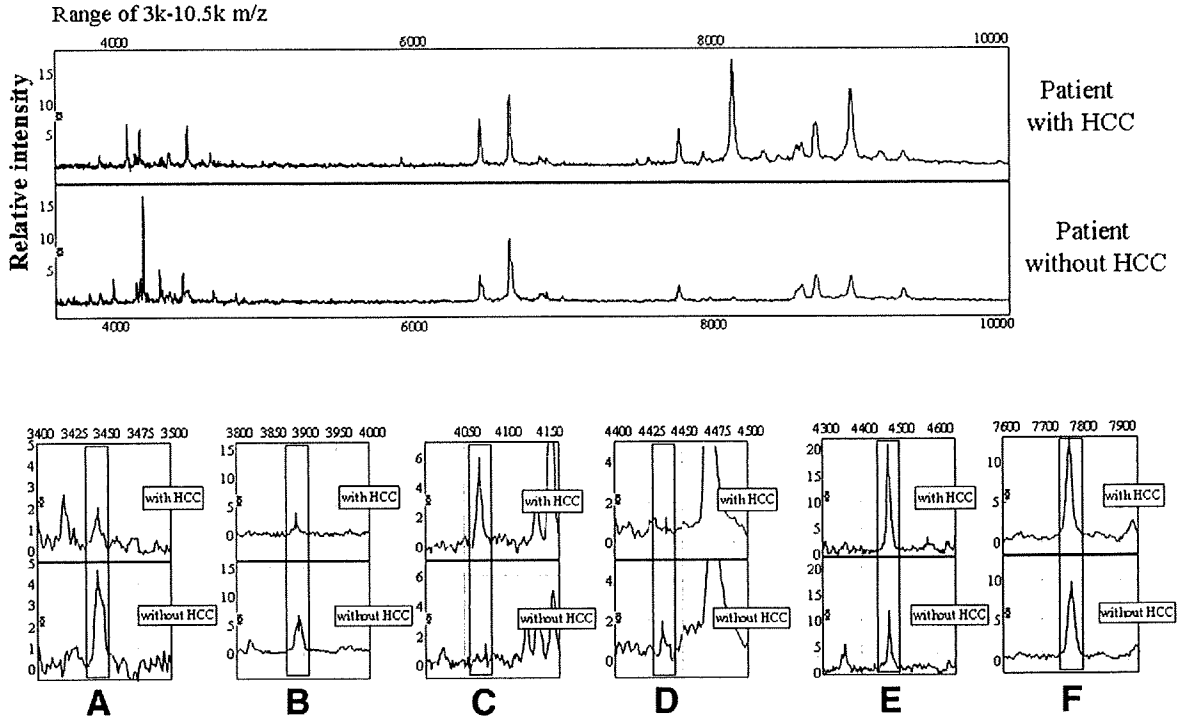

Fig. 1. Analysis of sera from patients with and without HCC (spectrum). Serum samples were applied to CM10 ProteinChip Arrays. Representative spectra from patients in each of the 2 groups (HCC and non-HCC) are presented. The horizontal axis indicates protein mass to charge (m/z), whereas the longitudinal 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 (F) 7770 m/z are shown.

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-

Table 3. Discriminatory Peaks and Mean Values Between Groups (HCC* and Non-HCC Group)

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.

munodepletion techniques, leading to the loss of valuable diagnostic information.[31] Therefore, we did not remove major serum proteins (albumin and IgG) from this study; analysis using the SELDI ProteinChip system can be performed without immunodepletion.

The characteristics of patients such as sex and age, sample collection method, processing and storage of samples, and data analysis methods may induce bias into proteomics-based biomarker discovery attempts. Because HCC occurs more frequently in males than females, we developed our classification model using male patients only. As a result, our study was not designed to address the benefit of our classification model for females with HCC. Villanueva et al.,[32] however, reported that gender did not appear to affect the peptide profile. We also evaluated five female patients with HCC; the peak intensity at 8136 m/z was elevated to a similar degree as that seen in male patients with HCC. Currently, a prospective study of female patients with or without HCC is underway to validate the utility of this classification model as a marker for the detection of HCC, particularly at early stages.

We demonstrated that 18 of the selected 55 protein peaks within a m/z range of 3000 to 10,500 range differed between patients with and without HCC by univariate analysis. Based on the peak intensities of the 55 peak proteins, 6 peaks were selected to construct the decision tree for the first analysis group using Biomarker Patterns Software and a 10-fold cross-validation approach. Two (3444 and 3890 m/z peaks) of those 6 peaks, however, were not significantly different between the HCC and non-HCC groups by univariate analysis (P values of 0.2, Table 3). The selection process to construct the decision tree was not based on univariate analysis; the presented decision tree was developed using multivariate binary logistic regression to determine the peaks best able to differentiate patients with and without HCC.[19,33] In fact, the ROC AUC of each of these 6 peaks were between 0.61 and 0.71, which tended to be more discriminatory than other serum markers. The decision tree proved to be best able to predict the presence of HCC in comparison with other serum markers. For these reasons, analysis of all 6 peaks, including the 2 peaks that were not significantly different between patients with and without HCC (peaks at m/z = 3444 and 3890), had the highest discriminatory power.

The algorithm used in this study is well established as a diagnostic tool for malignant neoplasms.[13,16,34,35] In comparison with the use of a single biomarker for the diagnosis of disease, multiple-biomarker analysis has both higher sensitivity and specificity. Indeed, our multimarker analysis was more accurate than existing tumor marker analysis methods (Table 4). Multimarker analysis is useful to predict HCC in patients with liver cirrhosis, which has high malignant potential and heterogeneous characteristics. Complex serum proteomic patterns may reflect the underlying pathological state of an organ, including

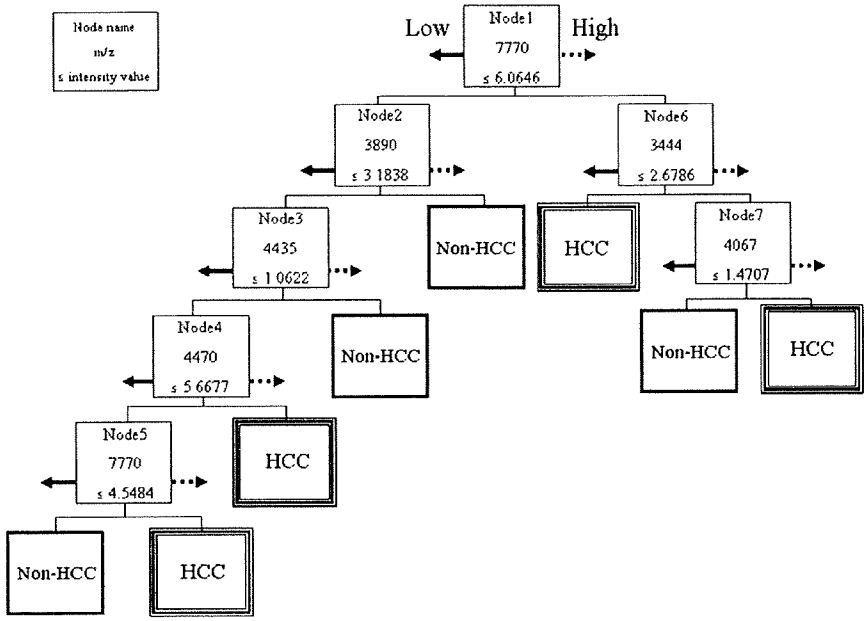

Fig. 2. Classification of HCC and non-HCC samples in the first analysis group. The decision tree was constructed using serum samples from 79 patients. The classification of a particular pattern began at the roof node, following the appropriate links based on the answer to the question at each node. If the peak 1 intensity was higher, the right node was selected. If the peak 1 intensity was lower or equal, the left node was selected. This process was repeated until a terminal node was reached. The decision tree was constructed to correctly classify 97% of the HCC samples in the first analysis group. The upper, middle, or lower lines in the box indicate the node name, molecular weight, and intensity value, respectively.

HCC. Recently, Schwegler et al.[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 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).[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 2 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.[16,36]

Although serum AFP level greater than 400 ng/ml serves as a useful method for the diagnosis of HCC,[37] this detection method is insufficiently sensitive to detect small HCCs.[38] Although the utility of several other markers has been shown to be superior to AFP in detecting early HCC,[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.[26] 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.[26,41] Our analysis of the proteome using the SELDI technique demonstrates that this methods 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.,[42] 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.

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Transgenic expression of osteoactivin in the liver attenuates hepatic fibrosis in rats

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Abstract

The role of osteoactivin (OA) in liver fibrogenesis remains unclear. After feeding wild-type (WT) and OA transgenic (OA-Tg) rats a choline-deficient, L-amino acid-defined (CDAA) diet for 12 weeks, we evaluated liver fibrosis. Hepatic fibrosis and expression of α-smooth muscle actin protein in OA-Tg rats were reduced in comparison to WT rats. Our examination of the expression of 31,100 genes by microarray analysis identified 177 and 256 genes that were upregulated and downregulated, respectively, by at least twofold in OA-Tg rat livers in comparison to WT rat livers. Of these genes, we confirmed a significant downregulation in the expression levels of tissue inhibitor of metalloproteinase-1 and -2, type I collagen, and platelet-derived growth factor receptor-α and -β in the livers of OA-Tg rats. These results indicate that transgenic OA expression attenuates the development of hepatic fibrosis in association with the suppression of specific genes involved in its pathogenesis.

Keywords: Osteoactivin; Choline-deficient L-amino acid-defined diet; Hepatic fibrosis; TIMP; PDGF receptor

Hepatic fibrosis is a common response seen in chronic liver diseases, which ultimately leads to cirrhosis, a major public health problem worldwide. Hepatic fibrosis can be attenuated by treatment of the cause of liver injury such as anti-viral therapy and abstinence from alcohol [1]. There is no efficient treatment, however, for most causes of chronic liver disease and no effective direct treatment for hepatic fibrosis in a clinical setting.

Hepatic stellate cells (HSC) are currently thought to be primarily responsible for hepatic fibrosis. In response to hepatic injury, quiescent HSCs are activated to become myofibroblastic cells, which produce cytokines and matrix proteins like transforming growth factor (TGF)-β and tissue inhibitor of matrix metalloproteinase (TIMP)-1 [2]. In addition, the signal transduction pathways activated in HSC by hepatic injury have suggested targets for the direct treatment of hepatic fibrosis in animal models [3,4].

A wide spectrum of pathological features are observed in non-alcoholic fatty liver disease (NAFLD), ranging from fatty liver to steatohepatitis and hepatic fibrosis, and hepatocellular carcinoma (HCC). In the choline-deficient, L-amino acid-defined (CDAA) diet rat model, liver steatosis occurs within one week. Hepatic fibrosis appears one month after administration of CDAA diet, with cirrhosis appearing after three or four months and HCC develops twelve to fifteen months after administration of CDAA [5]. To represent these pathological features, rats fed a CDAA

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diet is recognized one of the animal models for chronic liver disease, especially NAFLD.

Osteoactivin (OA) cDNA was originally isolated from osteopetrotic bone [6]. OA, also known as glycoprotein nonmetastatic melanoma protein B or dendritic cell-associated heparan sulfate proteoglycan-integrin ligand, is a type I transmembrane glycoprotein that influences the adhesion and migration of select cell types, including fibroblasts [7]. In addition, we previously reported the OA gene as a molecule that is differentially expressed in the livers of rats administered a CDAA diet [8]. The molecular mechanism by which OA functions in liver disease, however, has yet to be fully clarified. This study sought to determine the role of OA in hepatic fibrosis using transgenic rats that express OA in the liver after CDAA diet-induction of hepatic fibrosis.

Materials and methods

Generation of transgenic rat. A rat OA cDNA fragment, encoding the entire open reading frame from nucleotide 110 to 1917, was amplified by polymerase chain reaction (PCR) and cloned into the *Eco*RI site of the pLG-1 expression vector, which contains the human serum amyloid P (SAP) promoter and a rabbit β-globin non-coding exon/intron [9]. After digestion of the resulting plasmid with *Hin*dIII and *Xho*I, the 3.8-kb SAP-OA gene fragment was microinjected into fertilized Sprage Dawley (SD) rat eggs to produce transgenic rats (OA-Tg rats). Animal protocols were approved by the ethical committee of the Faculty of Medicine, University of Miyazaki.

RNA isolation, Northern blotting and RT-PCR. The total RNA was extracted from liver tissue, separated on agarose gels and transferred onto nylon membranes. To detect the OA mRNA transcript, we used radiolabeled 1808-bp rat OA cDNA and 483-bp rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA as probes. We also validated the OA gene expression levels by semi-quantitative reverse transcription (RT)-PCR. The total RNA was reverse transcribed using Molony murine leukemia virus reverse transcriptase (TaKaRa, Tokyo, Japan) in the presence of random hexamers. The following primers were then used: 5′-ACACTG CAGCCTGACAACTCA-3′ and 5′-TTTGCCCTTGACCACGTTC-3′ for rat OA and 5′-ACTCTACCCACGGCAAGTTCA-3′ and 5′-GG CAGTGATGGCATGGACT-3′ for rat GAPDH. The reverse-transcribed mixture was amplified by PCR in a 25 μl volume. PCRs were initially denatured, then cycled at 94 °C for 30 s, 52 °C (OA) or 59 °C (GAPDH) for 30 s, and 72 °C for 30 s. Thirty cycles served to amplify OA or GAPDH. PCR products of OA and GAPDH were examined by agarose gel electrophoresis and visualized with ethidium bromide. Densitometric analysis examined the amount of PCR products semi-quantitatively by measuring absorbance on a Bio-1D apparatus (M&S Instruments Trading Inc., Tokyo, Japan).

Hepatic fibrosis induced by a 12-week CDAA diet. Ten-week-old male SD rats bearing or lacking the SAP-OA gene (OA-Tg and WT, respectively) were used. SD rats were obtained from Japan SLC (Yokohama, Japan). After at least a one-week acclimation period on a standard diet, OA-Tg and WT (control) rats were switched to the CDAA diet (Dyets, PA) as a model of hepatic fibrosis. Rats were analyzed after a 12-week administration of the CDAA diet.

Histological and immunohistochemical analysis, and quantification of hepatic hydroxyproline content. Tissue samples were fixed in 10% phosphate-buffered formaldehyde, then embedded in paraffin and stained with either Azan or Sirius Red. Three liver fragments (>1 cm^2 each) were randomly taken from the right, median, and left lobes of each rat liver for morphometric studies. Sirius Red (Sirius Red 80; MUTO PURE CHMICALS Co., Tokyo, Japan) staining was performed as described previously [10]. To analyze fibrosis present in Sirius Red-stained sections, the red-stained areas were measured on a video-screen display in a blinded manner using a digital image analyzer pixs2000Pro (Inotech, Hiroshima, Japan) [4]. Three fields were selected randomly from each of three sections per sample; samples from six rats from each group were examined. Thus, a total of 54 fields were analyzed for each group. After signals were quantified, we calculated the mean area of fibrosis. Immunohistochemical analysis of α-smooth muscle actin (α-SMA) (Dako Japan, Kyoto, Japan) was performed and hepatic hydroxyproline content was determined as previously described [11,12].

DNA microarray analysis. RNA samples were reverse-transcribed and copied into dsDNA. *In vitro* RNA transcription was then performed to incorporate biotin-labeled ribonucleotides into the cRNA transcripts. The resulting cRNA samples were hybridized to a Rat Genome 230 2.0 Array (Affmetrix Inc., CA). Detailed protocols for the analysis of microarray data have been previously described [13,14]. We excluded genes that were not expressed or those that were expressed at levels below the cutoff level for detection in both OA-Tg and WT rat livers. These data were transferred to GeneSpring software (Silicon Genetics, CA) for additional analysis.

Western blotting. Liver tissues were homogenized in Tissue Protein Extraction Reagent (Pierce Biotechnology, IL). Ten micrograms of sample was subjected to SDS–polyacrylamide gel electrophoresis and transferred onto Immobilon P membranes (Millipore Corp., MA). The following primary antibodies were used for analysis: monoclonal anti-α-SMA antibody (SIGMA), polyclonal anti-TIMP-1 antibody, polyclonal anti-platelet-derived growth factor receptor (PDGFR)-α antibody (Santa Cruz Biotechnology, CA) and monoclonal anti-β-actin antibody (Dako Japan). Bound antibody was detected with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Amersham Biosciences, Buckinghamshire, UK). Proteins were then visualized using the ECL Western blotting detection kit (Amersham).

Statistical analysis. Results are presented as means ± standard deviation. Statistical analysis was performed using Statview J-4.5 software (Abacus Concepts, Inc., CA). Differences were assessed by the Kruskal–Wallis analysis and/or the Mann–Whitney *U* test. The significance level was set at $P < 0.05$.

Results

Osteoactivin expression in the various organs

Northern blot analysis revealed that rat OA mRNA was strongly expressed in the lung and spleen and weakly expressed in the brain, heart, and liver. Expression was

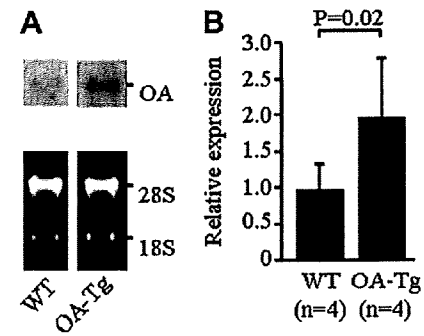

Fig. 1. Osteoactivin (OA) expression in the liver. (A) Northern blot analysis of OA expression in rat at 10 weeks of age. (B) Quantitative determination of OA mRNA using semiquantitative RT-PCR is shown as an average of four experiments ± standard deviation. The mean relative intensity in WT rat livers with normal diet administration was normalized to a value of 1.

Table 1
Serum biochemical markers in osteoactivin transgenic rats

	Normal diet normal rat ($n = 3$)	CDAA12W	
		WT ($n = 6$)	OA-Tg ($n = 6$)
Glucose (mg/dl)	157.3 (7.77)	217.2 (32.4)[a]	257.5 (68.8)[a]
ALT (IU/L)	65.7 (10.7)	53.0 (14.1)[a]	120.8 (30.1)[a,b]
LDH (IU/L)	1951.7 (501.3)	1173.2 (516.8)	1974.0 (811.3)
ALP (IU/L)	799.7 (44.0)	739.5 (331.6)	647.2 (129.5)
Triglyceride (mg/dl)	187.3 (64.0)	40.0 (8.44)[a]	105.5 (64.7)[c]
Total cholesterol (mg/dl)	70.7 (7.51)	62.5 (12.9)[a]	100.2 (13.1)[a,b]

Results (and standard deviation of the mean) from 6 rats/group at the end of feeding period were shown. WT, wild-type; OA-Tg, osteoactivin transgenic.
[a] $P < 0.05$ versus the normal rat with normal diet.
[b] $P < 0.01$ versus WT rats with CDAA diet.
[c] $P < 0.05$ versus WT rats with CDAA diet.

absent from the kidneys of non-transgenic littermates and SD rats as previously reported [8]. The expression levels of OA in the lung, spleen, brain, and heart of OA-Tg rats were similar to those seen in non-transgenic littermates (data not shown). In contrast, the OA expression in the liver of OA-Tg rats were higher than those seen in non-transgenic littermates, and the levels in those was twofold higher by semi-quantitative RT-PCR analysis (Fig. 1).

Attenuation of CDAA diet-induced hepatic fibrosis in OA-Tg rats

Serum levels of ALT, triglyceride, and total cholesterol in OA-Tg rats were significantly higher than those seen in WT rats (Table 1). In contrast, the serum levels of glucose, LDH and ALP did not significantly differ between OA-Tg and WT rats.

Hepatic fibrosis was induced in both OA-Tg and WT rats by a 12-week CDAA diet administration. Histological analysis with Azan and Sirius Red staining demonstrated CDAA-induced severe fibrosis in the livers of WT rats (Fig. 2A and C). In contrast, fibrosis was not as prominent in the livers of CDAA-treated OA-Tg rats (Fig. 2B and D). This reduction in the severity of the fibrosis was observed in all areas of the liver, with no significant differences noted between the different lobes. In addition, the number of CDAA-induced fibrosis areas and the hydroxyproline content of OA-Tg rat livers were significantly lower in comparison to those seen in WT rats (Fig. 2E and F).

Overexpression of OA decreased the number of activated hepatic stellate cells

Although the number of activated HSCs expressing α-SMA (a marker of activated HSC) increased in WT rats fed the CDAA diet, overexpression of OA dramatically reduced the number of α-SMA-positive cells observed in

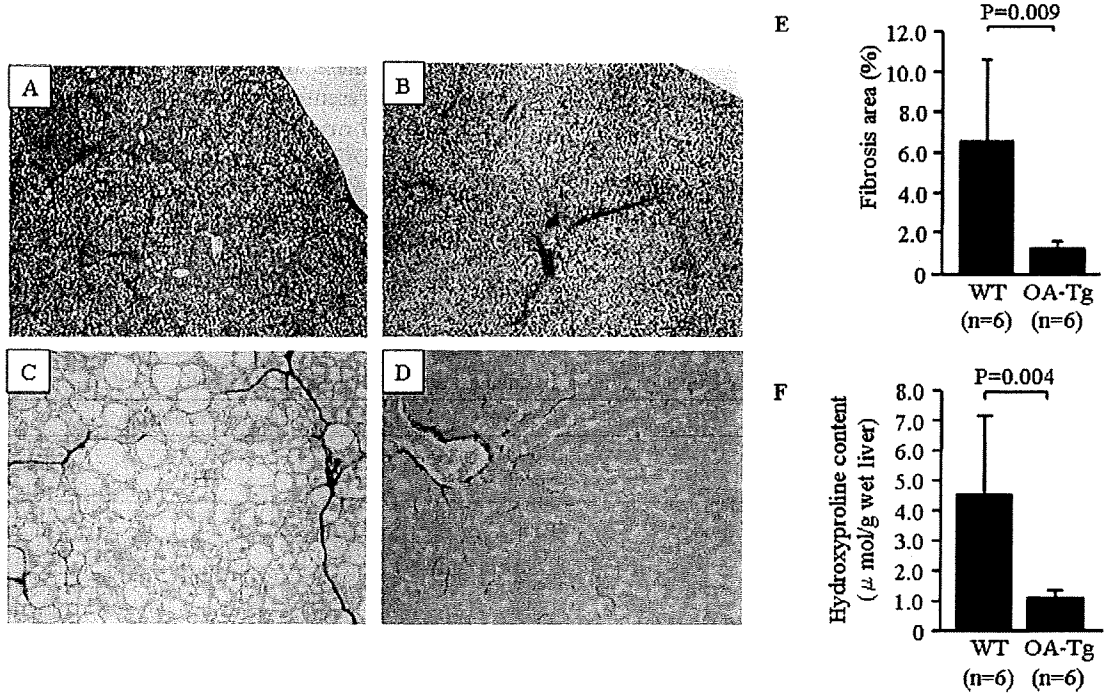

Fig. 2. Histological features of the liver in non-transgenic (A,C) or transgenic (B,D) rats after 12 weeks CDAA diet administration. Representative Azan (A,B) or sirius red (C,D) staining of the liver tissue (original magnification 40× (A,B), 100× (C,D)). In addition, quantitative evaluation of hepatic fibrosis in osteoactivin transgenic (OA-Tg) ($n = 6$) and non-transgenic (wild-type; WT, $n = 6$) rats are shown. (E,F) Morphometric quantification of the percentage of fibrosis areas and the hydroxyproline content in the livers of OA-Tg rats were significantly lower than those seen in WT rats, respectively.

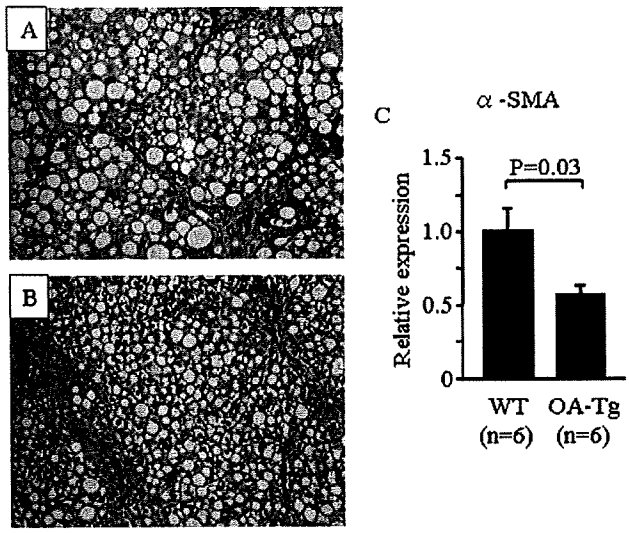

Fig. 3. Quantitative evaluation of hepatic α-smooth muscle actin (α-SMA) expression after 12 weeks CDAA diet administration. (A,B) Representative immunohistochemistry examining α-SMA expression in the livers of wild-type (A) or osteoactivin transgenic (B) rats (original magnification 100×). (C) Quantitative expression of hepatic α-SMA was determined by western blot analysis using an image analyzer. Results are shown as the averages with standard deviations. Expression of hepatic α-SMA in osteoactivin transgenic (OA-Tg) rats was significantly lower than that seen in wild-type (WT) rats.

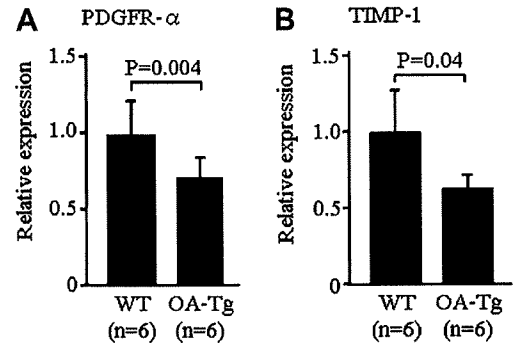

Fig. 4. Quantitative evaluation of hepatic platelet-derived growth factor receptor-α (PDGFR-α) and tissue inhibitor of metalloproteinase-1 (TIMP-1) expression after 12 weeks CDAA diet administration. (A,B) Quantitative expression of hepatic PDGFR-α and TIMP-1 were determined by Western blot analysis using an image analyzer. Results are shown as the averages with standard deviations. Expression of hepatic PDGFR-α (A) and TIMP-1 (B) in osteoactivin transgenic (OA-Tg) rats were lower than those seen in wild-type (WT) rats.

the liver (Fig. 3A and B). By western blot analysis, we also observed a significant decrease in α-SMA protein expression in OA-Tg rats in comparison to WT rats (Fig. 3C).

Gene expression profiles in the liver using DNA microarray analysis

The total RNA, isolated from the livers, of six OA-Tg or six WT rats, were mixed equally and hybridized in parallel to two identical oligonucleotide arrays. cRNA poles of OA-Tg or WT rats were used in each array. The statistical analysis identified 15692 transcripts which were expressed above the cutoff level for detection in both OA-Tg and WT rats. Of those genes, 177 transcripts displayed significantly increased signal intensities in mixed RNA derived from OA-Tg rat liver in compared to that from WT rat liver; 256 transcripts exhibited reduced signal intensities (data not shown). Of these genes, only 59 and 121 genes, respectively, have functional annotations in public databases. We selected from those 180 transcripts genes associated with fibrosis using the NetAffx Analysis Center database. Table 2 displays the numerical and descriptive analysis of these differentially expressed fibrosis genes; genes that presented with a fold change higher than 2.0 (upregulated) or lower than 0.5 (downregulated). Type I collagen, PDGF receptor (PDGFR)-α and -β, and TIMP-1 and -2, which are factors known to contribute to the development of hepatic fibrosis, were markedly downregulated in the livers of OA-Tg rats in comparison to WT rats. We also confirmed the downregulation of

Table 2
List of the fibrosis associating genes in osteoactivin transgenic rat after the administration of 12 weeks CDAA diet[a]

Gene name	Symbol	Fold change over controls	Accession No.
Collagen, type 1, alpha 1	Col1a1	0.225	BI285575
Suppressor of cytokine signaling 2	Socs2	0.359	NM_058208
Latent transforming growth factor beta binding protein 1	Ltbp1	0.38	NM_021587
Platelet derived growth factor receptor, alpha polypeptide	Pdgfra	0.391	AI232379
Tissue inhibitor of metalloproteinase 2	Timp2	0.397	BM388843
Serine proteinase inhibitor, clade H, member 1	Serpinh1	0.399	BI285495
Collagen, type III, alpha 1	Col3a1	0.406	BI275716
Tissue inhibitor of metalloproteinase 1	Timp1	0.406	NM_053819
Hypothetical gene supported by NM_031525	Pdgfrb	0.436	BM389426
Prostaglandin E receptor 1	Ptger1	0.467	AA945828
Fibrillin 1	Fbn1	0.478	BM389019
Serine protease inhibitor, Kazal type 1	Spink1	2.522	NM_012674

[a] Selected genes associated with fibrosis those are altered in osteoactivin transgenic (OA-Tg) rat. Fold changes in the livers of OA-Tg rats are compared to those in the livers of wild-type rats after a 12-week administration of the CDAA diet. Prior to DNA microarray analysis, mRNAs were mixed from each group ($n = 6$, each) as described in Materials and methods.

PDGFR-α mRNA expression in the liver by RT-PCR (data not shown). In addition, the expression of PDGFR-α and TIMP-1 in the liver observed by Western blot analysis was clearly decreased in the OA-Tg rats in comparison to WT rats (Fig. 4).

Discussion

Expression of the rat OA gene restricted to osteoblasts in bone [6]. Haralanova-Ilieva et al. demonstrated that OA is expressed at high levels in normal and inflammatory liver macrophages, suggesting a role for this protein in acute liver injury [15]. OA is also expressed in dendritic cells and tumor cells. OA is thought to induce fibroblasts activation [16]. OA overexpression increases the invasiveness and metastatic potential of rat hepatoma cells both *in vitro* and *in vivo* [8]. Although these results indicate that OA has multiple effects on different cell types, the function of OA in hepatic fibrosis remains unclear. In this study, we provide the first direct evidence that transgenic expression of OA in the liver inhibited hepatic fibrosis in rats fed a CDAA diet for 12 weeks.

Transgenic expression of OA was driven by the SAP promoter, which induces specific gene expression in hepatocytes, but not non-parenchymal cells [17]. Although transgenic expression of OA was only induced by the SAP promoter at low levels, hepatocyte expression of OA in OA-Tg rats was able to reduce hepatic fibrosis in association with a decrease in the number of activated HSCs. HSC activation typically induces a myoblastic, fibroblastic phenotype of these cells. Overexpression of OA in fibroblasts, but not mouse myoblasts, induced the expression of matrix metalloproteinase (MMP)-3. Thus, OA may function as an activator for fibroblasts that have infiltrated denervated skeletal muscle [16]. In this study, however, transgenic expression of OA had no effect on MMP-3 expression in the liver (data not shown). OA may have different roles in liver HSCs and muscle fibroblasts. OA may induce different effects in different disease processes, such as hepatic fibrogenesis and denervation.

No apparent changes in matrix-related gene expression have been demonstrated in OA transgenic mice [16]. OA-Tg rats did not display any apparent abnormalities. After taking the CDAA diet for 12 weeks, however, serum ALT and total cholesterol (TC) levels were higher in OA-Tg rats than those seen in WT rats. Although ALT increases with increasing severity of hepatitis, it is not indicative of hepatic fibrosis severity; high levels of ALT are instead observed in the early phases of liver disease in rats fed a CDAA diet [8]. Decreasing levels of TC have been linked to increasing severity of liver disease [18], suggesting that low ALT and TC levels in WT rats fed a CDAA diet indirectly indicate the severity of hepatic fibrosis. Analysis of OA activity in the different stages of fibrosis is required to identify the molecular foundation of this effect on fibrotic pathogenesis.

OA, which localizes to the cell surface and lysosomal membranes [7], can also be secreted from cells [6]. The role of OA expression in chronic liver disease remains unclear; in humans, OA expression is not detectable in normal liver tissue [8]. OA transcripts become detectable in cirrhotic non-tumorous liver tissue that surrounds HCC foci. OA mRNA expression is strongly induced in the livers of rats fed the CDAA diet for 4–12 weeks. Additional exogenous expression of OA, however, attenuates hepatic fibrosis. Although we did not identify the cells expressing OA and could not evaluate the amount of OA secreted, the secreted form of OA released by hepatocytes may negatively regulate activated HSCs. Further examination, including the effect of secreted OA on the activation of non-parenchymal cells, is required.

Both PDGFR-α and PDGFR-β were downregulated by exogenous OA expression (Table 2). Expression of the PDGFR correlates well with the extent of hepatic fibrosis [19]. While PDGFR-α is constitutively expressed in quiescent HSC, PDGFR-β expression is induced as cells undergo myofibroblastic changes [20]. Although the data concerning PDGFR-α and PDGF-β expression patterns are conflicting [21], targeting PDGFR-α signaling is an attractive potential therapeutic intervention in hepatic fibrosis. We also demonstrated the downregulation of suppressor of cytokine signaling (SOCS)-2, latent transforming growth factor-β binding protein (LTBP)-1, prostaglandin E receptor 1, and fibrillin 1 in OA-Tg rat livers in comparison to WT rat livers. TGF-β activity requires the proteolytic cleavage of LTBP, a microfibril-associated protein that interacts with fibrillin [22]. A number of (myo)fibroblastic cell subpopulations in the liver synthesize fibrillin-1, whose expression is induced by TGF-β1. These results indicate that the attenuation of hepatic fibrosis by transgenic OA expression is closely linked to the suppression of these genes.

The attenuation of PDGF signaling in the livers of OA-Tg rats would theoretically be accompanied by decreases in the expression of multiple target genes of PDGF signaling in comparison to WT rats. In this study, expression of extracellular signal-regulated kinase (ERK)-7, one of the targets of PDGF signaling, in OA-Tg rat liver was below detectable levels, excluding ERK-7 from the analysis. ERK-7 levels, however, were decreased twofold in the livers of OA-Tg rats in comparison to WT rats. These results suggest that transgenic expression of OA in the liver functionally attenuates PDGF signaling. Despite the many differences at the mRNA level identified by cDNA array analysis, our study only examined a small fraction of the liver transcriptome; additional important genes may not have been identified in our analysis. Further analysis of mRNA samples derived from specific cell populations, such as hepatocytes, HSCs, Kupffer cells, and endothelial cells, from both OA-Tg and WT rats may be required.

In conclusion, transgenic rats expressing OA exclusively in hepatocytes exhibited attenuated hepatic fibrosis in response to a CDAA diet. The potential of OA to increase