**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* ($X10^4$/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* ($X10^4$/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* ($X10^4$/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

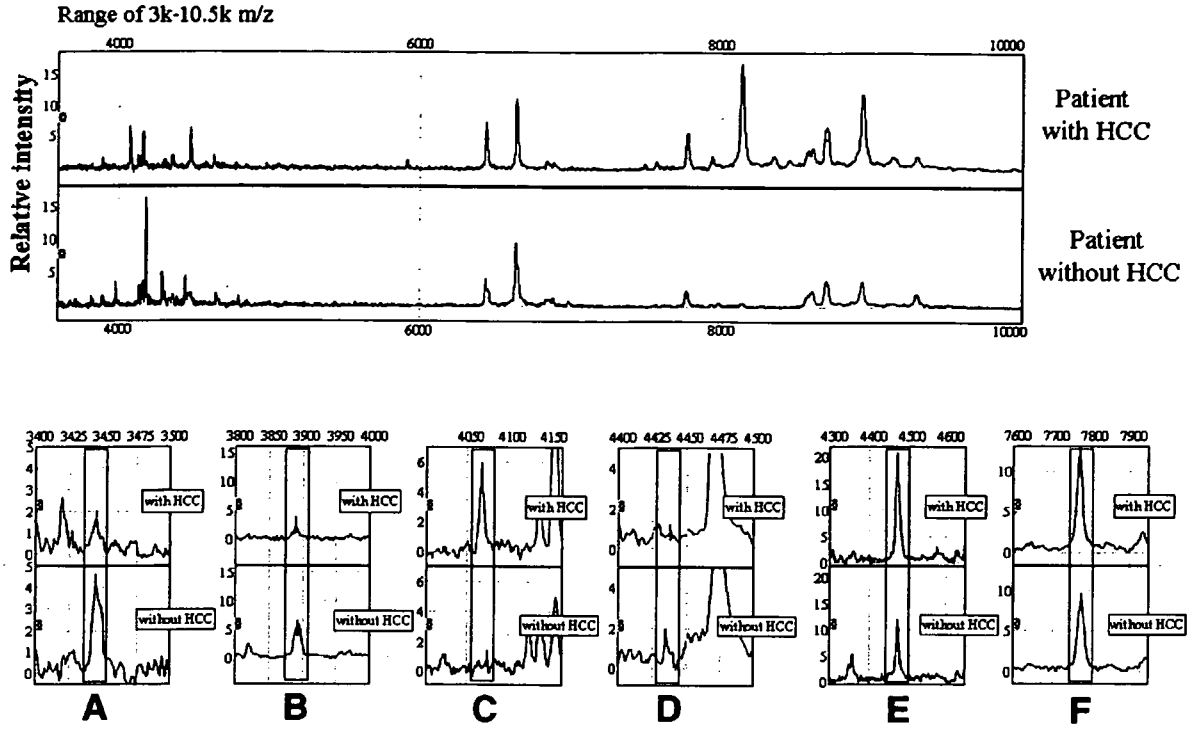


Fig. 1. Analysis of sera from patients with and without HCC (spectrum). Serum samples were applied to CM10 ProteinChip Arrays. Representative spectra from patients in each of the 2 groups (HCC and non-HCC) are presented. The horizontal axis indicates protein mass to charge (m/z), whereas the longitudinal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

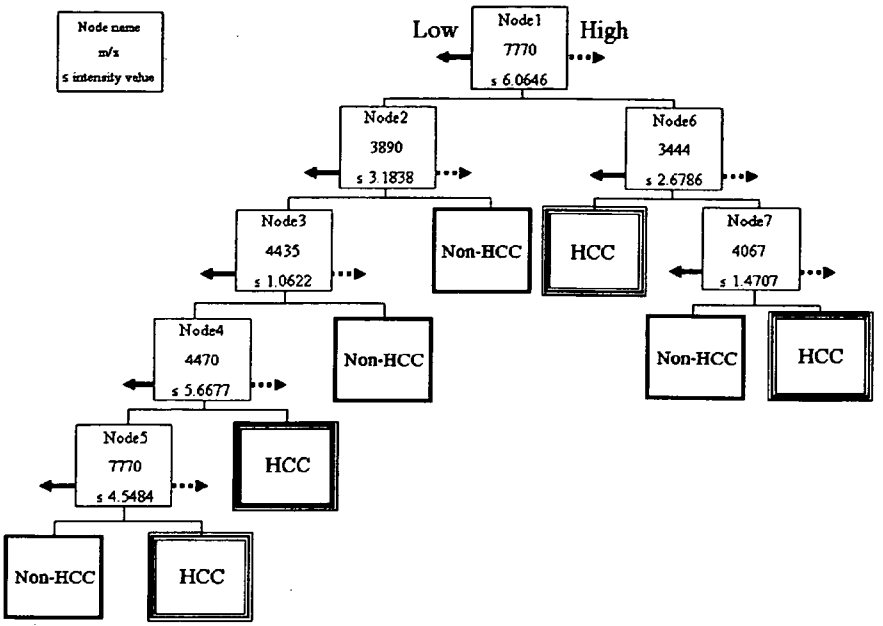


Fig. 2. Classification of HCC and non-HCC samples in the first analysis group. The decision tree was constructed using serum samples from 79 patients. The classification of a particular pattern began at the roof node, following the appropriate links based on the answer to the question at each node. If 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

Hiroo Abe [a], Hirofumi Uto [b], Yoichiro Takami [c], Yuka Takahama [c], Satoru Hasuike [a], Mayumi Kodama [a], Kenji Nagata [a], Akihiro Moriuchi [b], Masatsugu Numata [d], Akio Ido [d], Hirohito Tsubouchi [b,d,*]

[a] *Gastroenterology and Hematology, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan*
[b] *Department of Digestive and Life-style Related Disease, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan*
[c] *Miyazaki Prefectual Industrial Support Foundation, Miyazaki, Japan*
[d] *Department of Experimental Therapeutics, Translational Research Center, Kyoto University Hospital, Kyoto, Japan*

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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

* Corresponding author. Address: Department of Digestive and Life-style Related Disease, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan. Fax: +81 99 274 3504.
*E-mail address:* htsubo@m2.kufm.kagoshima-u.ac.jp (H. Tsubouchi).

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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

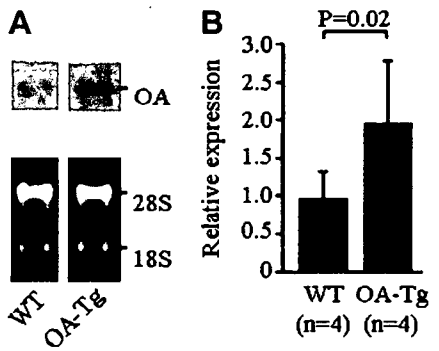


Fig. 1. Osteoactivin (OA) expression in the liver. (A) Northern blot analysis of OA expression in rat at 10 weeks of age. (B) Quantitative determination of OA mRNA using semiquantitative RT-PCR is shown as an average of four experiments ± standard deviation. The mean relative intensity in WT rat livers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

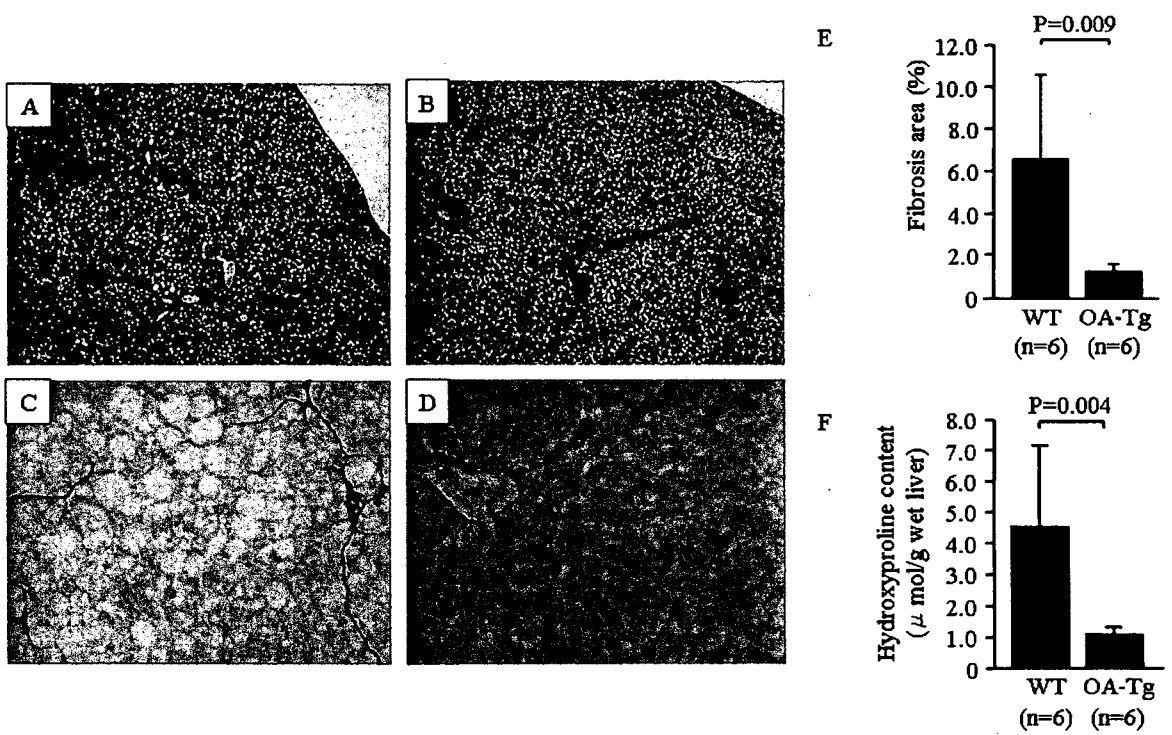


Fig. 2. Histological features of the liver in non-transgenic (A,C) or transgenic (B,D) rats after 12 weeks CDAA diet administration. Representative Azan (A,B) or sirius red (C,D) staining of the liver tissue (original magnification 40× (A,B), 100× (C,D)). In addition, quantitative evaluation of hepatic 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.

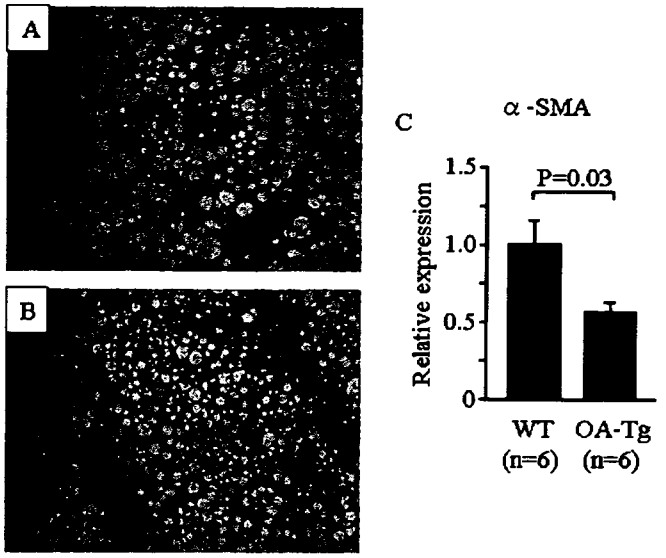


Fig. 3. Quantitative evaluation of hepatic α-smooth muscle actin (α-SMA) expression after 12 weeks CDAA diet administration. (A,B) Representative immunohistochemistry examining α-SMA expression in the livers of wild-type (A) or osteoactivin transgenic (B) rats (original magnification 100×). (C) Quantitative 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.

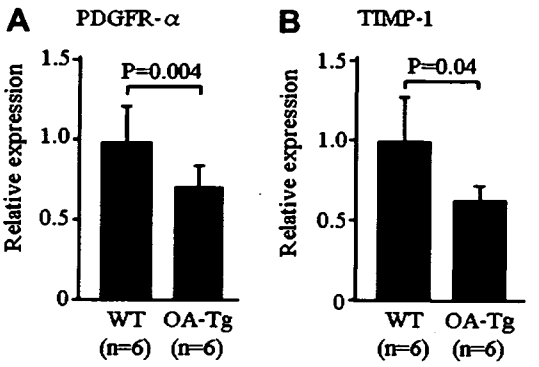


Fig. 4. Quantitative evaluation of hepatic platelet-derived growth factor receptor-α (PDGFR-α) and tissue inhibitor of metalloproteinase-1 (TIMP-1) expression after 12 weeks CDAA diet administration. (A,B) Quantitative expression of hepatic PDGFR-α and TIMP-1 were determined by Western blot analysis 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

the risk of HCC tumor invasiveness and metastasis may limit the use of this molecular target in the treatment of liver cirrhosis. Further investigation using a secreted form of OA will be necessary and may lead to the development of novel therapeutic approaches to hepatic fibrosis.

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# C型肝炎治療up to date

# C型慢性肝炎
## −ALT持続正常者の治療戦略−

宇都浩文（鹿児島大学大学院医歯学総合研究科消化器疾患・生活習慣病学講師）
桶谷　真（鹿児島大学大学院医歯学総合研究科消化器疾患・生活習慣病学講師）
井戸章雄（鹿児島大学大学院医歯学総合研究科消化器疾患・生活習慣病学准教授）
坪内博仁（鹿児島大学大学院医歯学総合研究科消化器疾患・生活習慣病学教授）

☞ *Point*

- ALT持続正常（persistent normal ALT；PNALT）の定義は明確ではないが、1年以上の経過でALTが3回以上すべて30IU/L以下で、血小板数が15万/μL以上、がPNALTとして適切と考えられる。
- HCVキャリアではALT値は肝細胞がん発症と関連し、ALTが低値を持続すれば肝細胞がん発症は少ないが、PNALTであっても経過観察中にALTが上昇するリスクは高い。
- PNALTの多くは、軽度ではあっても炎症や線維化を伴う肝組織を呈する。
- PNALTに対するIFN単独療法は効果が低いが、PEG-IFN/リバビリン併用療法は1型には48週間投与、2型には24週間投与でALT異常者と同等の効果が期待できる。
- 治療に伴う副作用や個人の医療費負担も考慮し、期待できる治療効果や治療に対する意欲なども踏まえて、PNALTの治療を判断すべきである。

C型肝炎ウイルス（hepatitis C virus；HCV）感染が持続すると、慢性肝炎を経て、肝硬変に進展し、肝細胞がんを発症するが、すべてのHCV持続感染者が重篤な肝疾患に進展するわけではない。病態の進展を予測するマーカーとしてアラニンアミノトランスフェラーゼ（alanine aminotransferase；ALT）値は非常に簡便で有用なマーカーであり、ALT正常が持続すれば肝線維化の進展は緩徐で、肝がん発症のリスクは低い。しかし、ALT持続正常症例のなかには肝病態が進展している例や、経過中にALTが上昇し、病態が悪化する症例も存在する。さらに、ALT持続正常症例に対する抗ウイルス効果はALT高値例と同等であることも報告されている。本稿では、ALT持続正常者の病態と治療戦略について解説する。



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| | 血小板数15万以上 | 血小板数15万未満 |
|---|---|---|
| ALT 30IU/L以下 | 2～4ヵ月ごとに血清ALT値のフォロー。ALT異常を呈した時点で完治の可能性、発がんリスクを評価し、抗ウイルス療法を考慮。 | 線維化進展例がかなり存在する。可能なら肝生検を施行し、F2A2以上の例に抗ウイルス療法を考慮。 |
| ALT 31～40IU/L | 抗ウイルス療法の適応 | 慢性肝炎治療に順ずる |

**表1　血清ALT正常C型肝炎例への抗ウイルス治療ガイドライン**

(厚生科学研究費補助金 肝炎等克服緊急対策研究. B型及びC型肝炎ウイルスの感染者に対する治療の標準化に関する臨床研究(熊田班、平成18年度)より引用)

## 血清ALTの正常値

HCVキャリアの約25％はALT正常が持続し、肝炎の活動性は低く、肝硬変への移行は少ないと報告されている[1-5]。しかし、ALT正常者のほうが、より進行した肝線維化を呈するとの報告もある[6]。また、正常ALT値の定義は確立していない。最近、6,835例の献血者のALT値を検討し、HCV陽性者や非アルコール性脂肪性肝疾患の患者と区別するための正常値は男性30IU/L以下、女性19IU/Lであることが、Pratiらにより報告された[7]。また、厚生労働省の肝炎治療標準化研究班の岡上らの調査では、全国72大学病院の正常上限値が、25～50IU/Lと施設間に大きな差があることが明らかにされている。このような理由から、日本でのALT値正常C型肝炎症例に対する抗ウイルス治療ガイドラインでは、ALT値の基準値を30IU/L以下とし、血小板数なども加味して病態を把握し、治療方針を決定することが推奨されている(**表1**)。

## ALT持続正常(persistent normal ALT；PNALT)の定義と病態

ALT値の正常値だけでなく、PNALTの定義もコンセンサスは得られていない。PNALTを対象として肝線維化の程度を評価した報告を比較すると、PNALTの肝線維化の程度は、PNALTを定義した際のALT測定回数と経過観察期間に依存すると報告されている[8]。つまり、ALT測定回数が少ない(3回以下)もしくは経過観察期間が短い(6ヵ月以下)場合のPNALTには、F3/4が10％含まれるのに対して、4回以上かつ6ヵ月以上の条件を満たしたPNALTにはF3以上は3％しか含まれない(**図1**)。また、Okanoueらは ALT 30IU/L以下が3回以上、1年以上にわたり持続し、さらに、血小板数を15万以上とすることでALT低値例に含まれる可能性のある肝硬変を除外して、PNALTを定義している[9]。この定義では、PNALTの肝組織所見は、129例中F0は41例(31.8％)であり、F3以上の症例はない(**図1**)。このように、ALT持続正常者は、軽度の炎症や線維化を伴う肝組織を呈する可能性が高いものの、肝線維化進展例(F3以上)を含む可能性が少ないという観点から、OkanoueらのPNALTの定義は適切と考えられる。

## PNALTの自然経過

PNALT 880人の経過を平均22ヵ月間経過観察した報告では[10]、21.5％で経過観察中にALTが上昇している。またPNALTを12年経過観察し、22％が経過中にALT上昇をきたし、その上昇はすべて経過観察を始めて最初の4年間に生じたとする報告もある[1]。一方、Okanoueらは持続的にALT値が30IU/L以下で血小板数が15万以上のPNALTであっても、その後の5年間の経過観察では30IU/L以下を持続したものは少なく14％で、一過性にALT値が30IU/Lを超えたものは57％、ALT値が持続的に30IU/L以上になったものが29％であったと報告している。われわれの検討でも、ALT低値(ALT 34IU/L以下)が持続しても、経過中にALTが上昇する(ALT 35IU/L以上となる)可能性があり、ALT 19以

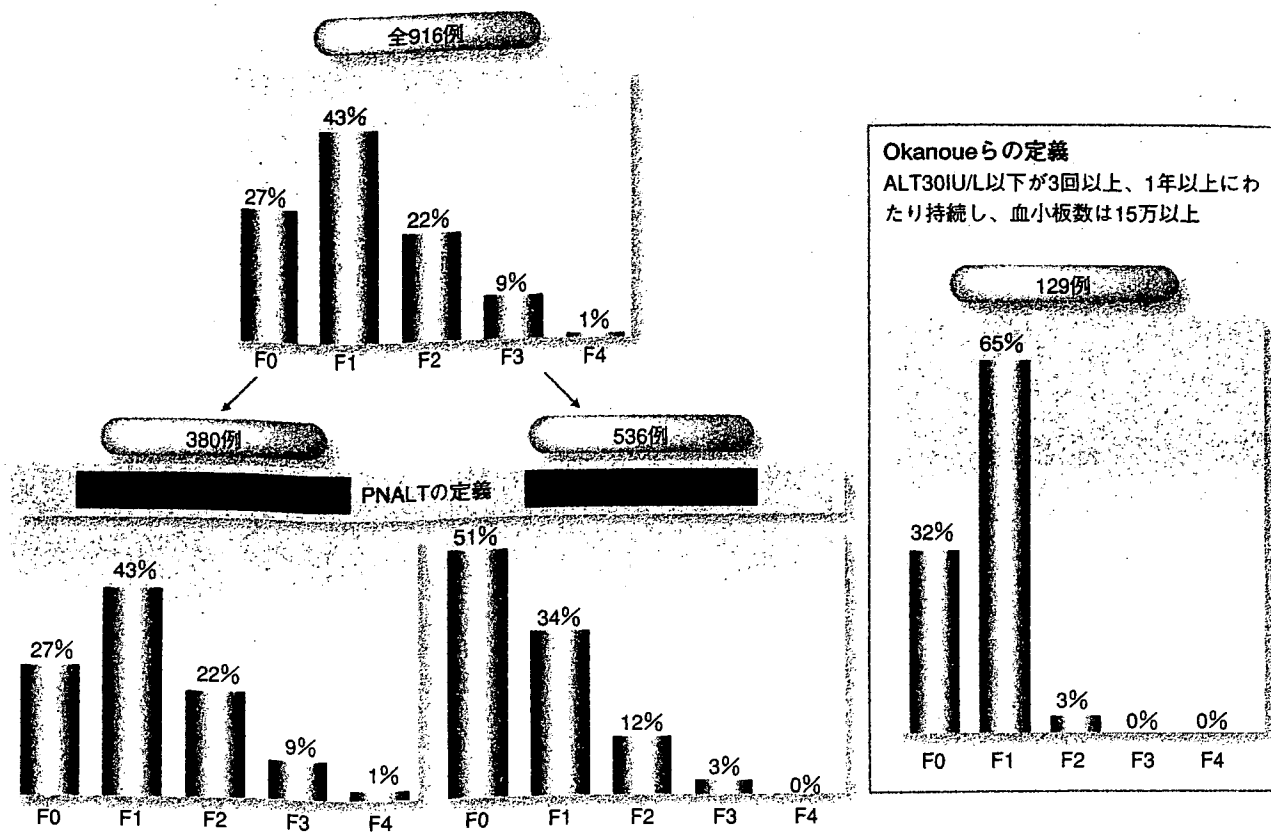


**図1　PNALTの定義と肝生検組織所見**(文献8、9から引用改変)

下よりもALT 20～34IU/Lのほうが ALTは上昇する可能性が高いことを明らかにした[11]。また、ALT正常者の肝線維化の進展速度はALT異常者に比して遅い。しかし、線維化速度は直線的ではなく、ALTが異常になれば、線維化の進展速度は増加する。

40歳時に肝機能正常か慢性肝炎かで2つのグループに分けて、その後の30年間の肝病態の進展を予測するモデルが報告された[12]。このモデルでは、ALT正常者は慢性肝炎よりも肝硬変や肝細胞がんに進展する可能性は低い。また、献血者におけるHCV抗体陽性者1,927名の検討では、ALTが20IU/L以下であれば肝細胞がんの発症はきわめて少ない[13]。われわれもHCV高感染地区住民検診の検討から、ALT値と肝細胞がん発症が強く関連することに加え(図2)、ALTが持続正常であれば、肝がん発症は非常に少ないことを明らかにした[14]。Okanoueらも PNALTを平均5年経過観察し、肝細胞がんの発症はなかったと報告している[9]。しかし、PNALTであっても、ALT上昇のリスクはあり[9,11]、ALT値が上昇すれば肝発がんのリスクは上昇することから、ALT持続正常者の定期的な経過観察が肝要である。また、治療適応については経過をみながら検討を重ねる必要がある。

## ALT持続正常者に対する抗ウイルス療法

PNALTの多くは予後良好であり、インターフェロン(interferon；IFN)単独治療の著効率が低いこと、IFN治療によって、ALT異常が出現することが危惧されることなどから、以前はPNALTに対する抗ウイルス療法は積極的には行われていなかった。しかし、

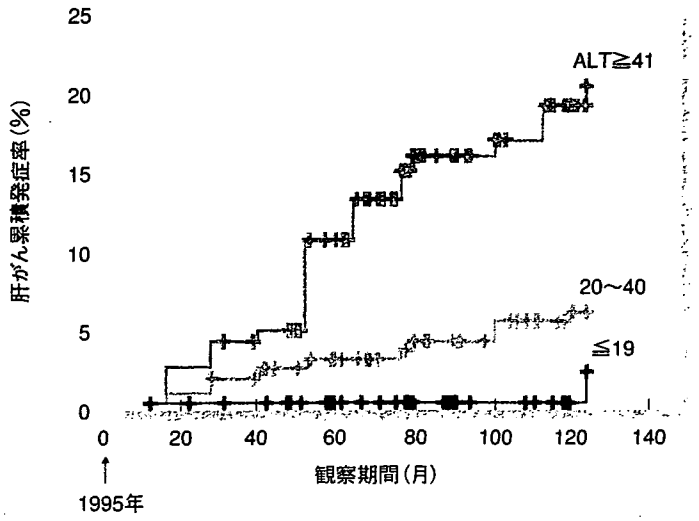


図2　HCV高感染地区住民HCV持続感染者における1995年のALT値とHCCの累積発生頻度

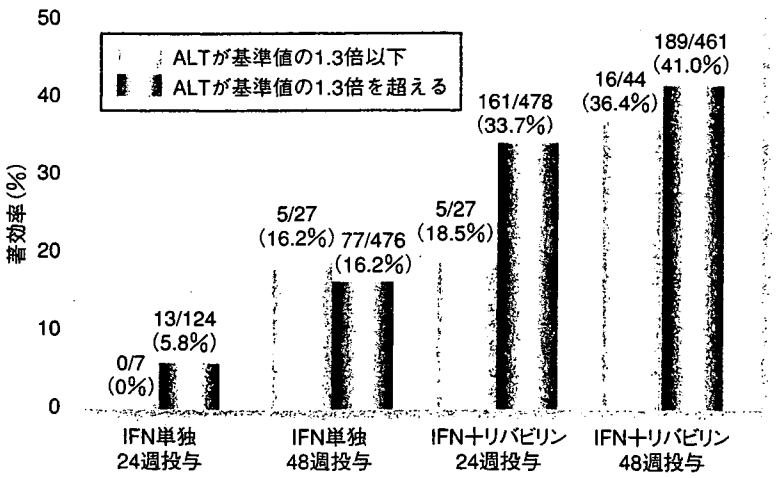


図3　治療前のALT値別にみたIFN単独もしくはIFNとリバビリン併用療法の著効率(文献15から引用改変)

ALTが基準値の1.3倍以下のC型慢性肝炎患者に対するIFNの48週間長期投与やIFNにリバビリンを併用投与することにより、著効率は向上することが報告された(**図3**)[15]。この報告では、IFNとリバビリン24週間投与併用療法では、ALTレベルにより著効率に差があるものの、48週間併用投与ではALTレベルとは関係しない。また、ZeuzemらはALT正常(30IU/L以下)のC型慢性肝炎患者に対するPEG-IFN/リバビリン併用治療効果を報告

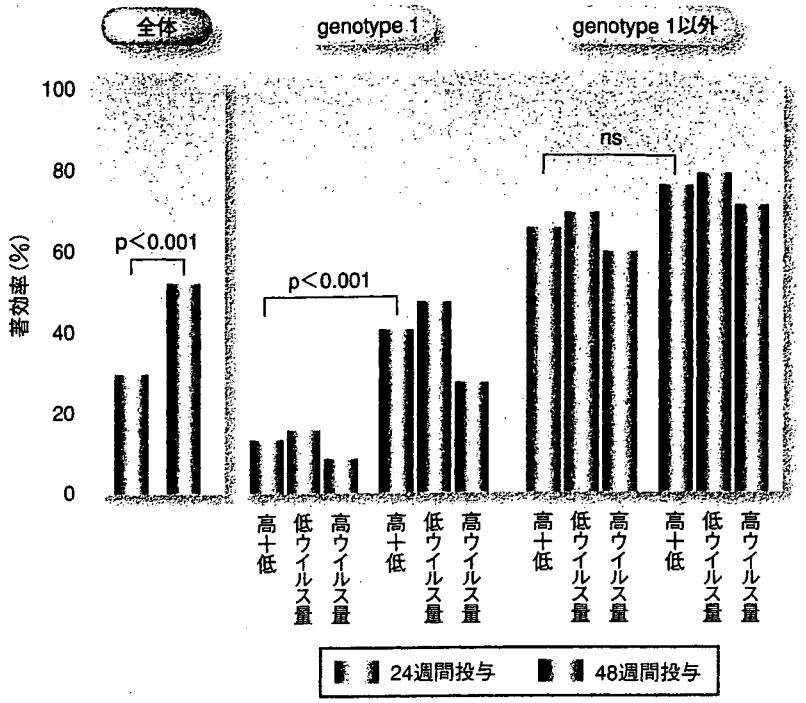


**図4　PNALTを呈するHCV感染者のgenotype別にみたPEG-IFNα2a＋リバビリン併用療法の著効率**(文献16を改変引用)

している(図4)[16]。48週間のPEG-IFNα2a/リバビリン併用療法では、1型で40%、1型以外で75%の著効率が得られ、1型以外では24週投与と48週投与で差がない。一方、1型では24週投与(著効率13%)より48週投与が著効率は有意に高く、無効群の治療後のALTの上昇は無治療群と差がない。さらに、PNALTに対するPEG-IFNα2bとリバビリンの併用療法にアマンタジンを追加し、12ヵ月間の効果を検討した成績では、著効率が63.5%であったことから[17]、PNALTに対するPEG-IFNαの効果は2aと2bではほとんど差がないと考えられる。このようなことから、PNALTのウイルス排除を目的とする場合には、1型では48週間、2型では24週間のPEG-IFN/リバビリン併用治療を行なうことが勧められる。

## 血清ALT正常C型肝炎例への抗ウイルス治療ガイドライン

厚生労働省治療標準化研究班(研究費補助金肝炎等克服緊急対策研究.B型及びC型肝炎ウイルスの感染者に対する治療の標準化に関する臨床研究(熊田班))から、血清ALT正常C型肝炎例への抗ウイルス治療ガイドラインが示された(表1)。このガイドラインでは、ALT値を30IU/L以下と31～40IU/Lまで、血小板数を15万/μL以上と15万/μL未満に分け、4つの群でそれぞれの治療指針を示している。ALTが基準値内(ALT30IU/L以下)であっても血小板数が低い場合(15万/μL以下)や、ALTが基準値に近い値(ALT 31～40IU/L)を示している場合は、肝炎が持続し、肝線維化が進展していることがあるため、遺伝子型、ウイルス量、年齢などを考慮し、抗ウイルス療法の適応を判断することが推奨されている。

最近の報告では、PNALTに対するPEG-IFN/リバビリン治療は、肝硬変のリスクを軽減し、コストの面からも治療が推奨されている[18]。さらに、PNALTで治療により著効が得られれば、self-administered Short Forum

(SF)-36 Health Survey とFatigue Severity Scaleで評価した生活の質(HRQoL; helth-related quality of life)は非著効より有意に改善すると報告された[19]。このような結果から、完治と病態進展抑制のためだけでなく、医療経済や生活の質を考慮しても、PNALTに対する抗ウイルス療法が積極的に行われつつある。しかし、治療に伴う副作用や個人の医療費負担も考慮し、期待できる治療効果や治療に対する意欲なども踏まえて、PNALTの治療を判断すべきである。

## おわりに

ALTの正常値やPNALTの明確な定義はないが、HCVキャリアではALT低値が持続すれば、肝線維化の進行や肝がん発症は少ないと考えられる。しかし、PNALTの治療を考えるうえでは、ALT値のみではなく血小板数や年齢なども考慮し、肝生検による肝疾患の重症度、予後、治療に伴う副作用、生活の質などを個々に検討し、治療するか判断すべきである。

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