Multilectin Assay for Detecting Fibrosis-Specific Glyco-Alteration by Means of Lectin Microarray

Atsushi Kuno, Yuzuru Ikehara, Yasuhiro Tanaka, Takashi Angata, Sachiko Unno, Maki Sogabe, Hidenori Ozaki, Kiyoko Ito, Jun Hirabayashi, Masashi Mizokami, and Hisashi Narimatsu

BACKGROUND: Despite the progress made in understanding glyco-alterations of specific glycoproteins such as α1-acid glycoprotein (AGP) associated with liver fibrosis, there has been no useful diagnostic assay with a lectin recognizing the fibrosis-specific alteration and an antibody against the core protein. We therefore developed a compatible multiple lectin-antibody sandwich immunoassay on the basis of the results obtained by the lectin microarray analysis for monitoring fibrosis.

METHODS: AGP-enriched fractions derived from 0.5-μL sera of 125 patients with staging-determined fibrosis (26.4% F0–F1, 25.6% F2, 24% F3, and 23.2% F4) were subjected to systematic analysis by antibody-overlay lectin microarray. Data were analyzed to statistically relate to the degree of fibrosis progression. Additionally, we applied an optimal lectin signal set on the microarray to distinguish 45 patients with cirrhosis from 43 patients with chronic hepatitis.

RESULTS: Signal patterns of the 12 selected lectins reflected fibrosis-associated glyco-alteration of AGP. Among the 12 lectins, we found a specific lectin at each stage of fibrosis (i.e., significant fibrosis, severe fibrosis, and cirrhosis) (P < 0.0001). The test for the detection of cirrhosis showed that combinational use of 3 lectins (AOL, MAL, and DSA) on the array enhanced the diagnostic value for liver cirrhosis to 95% diagnostic sensitivity and 91% diagnostic specificity.

CONCLUSIONS: The multiple lectin-antibody sandwich immunoassay targeting AGP enables monitoring of disease progression in chronic hepatitis patients at risk of developing hepatocellular carcinoma.

It is estimated that 350 million and 170 million people are chronically infected with the hepatitis B virus (HBV) and hepatitis C virus (HCV), respectively (1, 2). People with HBV or HCV have an increased risk of developing of chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Globally, 57% of cirrhosis cases are attributable to either HBV (30%) or HCV (27%), and 78% of hepatocellular carcinoma (HCC) cases are attributable to HBV (53%) or HCV (25%) (3). In Japan, >90% of HCC patients had chronic hepatitis associated with HBV (13%) or HCV (81%) during the period 1996–2000 (4). Although HCV infection is asymptomatic for about 10 years before chronic hepatitis develops, it causes cirrhosis and irreversible fibrosis during this interval. According to the association between staging fibrosis (F0–F4) and carcinogenesis, 7.9% of F4 stage fibrosis patients in Japan will develop HCC each year, whereas the corresponding rate for hepatitis patients with the primary F0/F1 stage is much lower (0.5%) (5). An accurate method for monitoring the progression of fibrosis is needed to identify F4 stage fibrosis patients as a high-risk group for the development of HCC, especially in the case of hepatitis C.

HCV patients are currently subjected to continuous monitoring for chronic inflammation by using biochemical markers, such as aspartate aminotransferase and alanine aminotransferase, to evaluate inflammatory activity and platelet count to assess the progression of fibrosis. However, platelet count is affected by physical status and some medical treatments. Consequently, despite the invasiveness and high risk, liver biopsies are regarded as the conventional method of evaluating the progression of fibrosis in patients with chronic hepatitis (6). As an alternative to liver biopsy, Imbert-Bismut et al. developed a method that involves.

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4 Nonstandard abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; AGP, α1-acid glycoprotein; AIST, National Institute of Advanced Industrial Science and Technology; PBS, phosphate-buffered saline; Triton X-100; ROC, receiver operating characteristic; AUC, area under the curve; LR, likelihood ratio.
analysis of a combination of biochemical markers (7, 8). Transient elastography (e.g., the Fibroscan), a noninvasive method based on physiological principles (9), has been considered to have a diagnostic value superior to that of the biochemical markers (10). However, transient elastography is not free of shortcomings (11), since its diagnostic success rate depends on the operator’s experience and the BMI of the patient. In fact, the American Association for the Study of Liver Diseases recommended that these noninvasive tests should not replace liver biopsies in routine clinical practice. Consequently, liver biopsy is widely regarded as the gold standard for defining liver disease status (12). Other more convenient assays using serum markers are still needed for frequent monitoring.

Recent omics-based technologies have facilitated the discovery of disease-specific biomarkers (13). We focused our attention on glycoproteomics, which involves analysis of the glycosylation patterns of glycoproteins, because almost all secreted proteins except albumin are eventually glycosylated. Fucosylation analysis of H9251/alpha-fetoprotein (theAFP-L3 fraction) improved the diagnostic specificity of this marker of HCC. As a result, the AFP-L3% test was approved by the U.S. Food and Drug Administration in 2005. More recently, several clinical glycomics technologies have been proposed for diagnosing liver cirrhosis and HCC (14–16).

In the present study, we applied an antibody-assisted lectin profiling technology to select an optimal lectin set for monitoring the progression of fibrosis (see Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol57/issue1) (17, 18). We focused on the glyco-alteration of alpha1-acid glycoprotein (AGP), a well-known acute-phase protein that is mainly secreted by the liver and of which glyco-alteration has been reported to occur during cirrhosis and during acute and chronic inflammation (19, 20).

### Materials and Methods

**SERUM SAMPLES AND CLINICAL DIAGNOSIS**

Since January 1999, we obtained long-term serial samples from 213 patients at Nagoya City University Hospital with chronic hepatitis B or C (21, 22). To optimize the procedure for the comparative assay of AGP glycosylation by means of lectin microarray, and to estimate the reproducibility, we collected control specimens from 20 randomly selected healthy volunteers aged 30–60 years. Exclusion criteria for healthy volunteers included hepatitis, other hepatic diseases, renal failure, clinical cardiovascular disease, and malignancies. The institutional ethics committees at Nagoya City University Hospital and the National Institute of Advanced Industrial Science and Technology (AIST) approved this study, and informed consent for the use of clinical specimens was obtained from all individuals before collection at the Nagoya City University Hospital and AIST. The general characteristics of the patient populations are presented in Table 1. Chronic hepatitis was defined as a persistent increase in serum alanine aminotransferase activity (>1.5 times the upper limit of the normal range [35 U/L] over a 6-month period, with at least 3 readings at 2-month intervals) accompanied by histological documentation of grading and staging scores (23). Fibrosis was graded according to the guidelines for fibrosis in the Histologic Activity Index by using biopsy or surgical specimens for pathological diagnosis. Biopsy specimens were classified as follows: F0, no fibrosis; F1, portal fibrosis without septa; F2, few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis. Three diagnostic targets in this study were defined as significant fibrosis: F2/F3/F4; severe fibrosis: F3 + F4; and cirrhosis: F4. Inflammation was also classified as follows: A0, no histological activity; A1, mild activity; A2, moderate activity; and A3, severe activity. Cirrhosis was also determined by ultrasonography (coarse liver architecture, nodular liver surface, and blunt liver edges) and evidence of hyper-

<table>
<thead>
<tr>
<th>Table 1. General characteristics of the patient population.</th>
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<tr>
<td><strong>n</strong></td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>F0–F1</td>
</tr>
<tr>
<td>F2</td>
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<td>F3</td>
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<td>F4</td>
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Data are means (SD) unless otherwise indicated.
spleenomegaly (ultrasonography) and/or a platelet count <100 000/mm³.

IMMUNOPRECIPITATION OF AGP FROM SERUM
We purified monoclonal anti-AGP IgG1 (clone AGP-47) from mouse ascites fluid (Sigma-Aldrich) by using a HiTrap Protein G HP column (GE Healthcare Bio-Science). We then biotinylated the obtained IgG1 with the Biotin Labeling Kit–NH₂ (Dojindo Laboratories) and used it as a biotinylated anti-AGP antibody. All sera were diluted 1:10 with PBS containing 0.2% SDS and then heated at 95°C for 20 min. The resulting solution (5 µL) was reacted with antibody solution (40 µL) containing 100 ng of the biotinylated anti-AGP antibody at 4°C for 2 h. After the incubation, we added 10 µL streptavidin-immobilized magnetic bead (Dynabeads MyOne™ Streptavidin T1; DYNAL Biotech ASA) conjugate to the reaction tube and incubated it at 4°C for 1 h with vigorous shaking. The beads, which captured AGP, were washed 3 times with 500 µL 1% Triton X-100 in TBS (TBSTx), and then the bound AGP was eluted with 10 µL of the elution buffer (TBS containing 0.2% SDS) by heat denaturation at 60°C.

ANTIBODY-OVERLAY LECTIN MICROARRAY
The purified proteins were diluted to 60 µL with PBS containing 1% Triton X-100 (PBSTx) and then applied to the lectin array containing triplicate spots of 43 lectins as described previously (24). After incubation at 20°C for 12 h or longer, an excess amount of blocker glycoprotein (20 µg nonlabeled human serum polyclonal IgG) was added to the glass slide before detection by the biotinylated anti-AGP antibody. After incubation at 20°C for 30 min, the reaction solution was discarded and the glass slide was washed 3 times with PBSTx. A 60-µL volume of biotinylated antibody solution in PBSTx was applied to the array, which was then incubated at 20°C for 1 h. After 3 washes with PBSTx, 60 µL Cy3-labeled streptavidin (GE Healthcare Bio-Science) solution in PBSTx was added to the array and incubated at 20°C for 30 min. The glass slide was rinsed with PBSTx and scanned by using an evanescent-field fluorescence scanner (Glycostation™; Moritex). We analyzed all data by using the Array Pro Analyzer, version 4.5 (Media Cybernetics). The net intensity value for each spot was calculated by subtracting the background value from the signal intensity values of 3 spots. We selected scanning data under appropriate gain conditions, which provided the intensities of all positive spots <40 000. The relative intensities of positive lectins were determined from their ratio to the fluorescent intensity of an internal-standard lectin DSA.

RESULTS

DIFFERENTIAL GLYCAN ANALYSIS OF SERUM AGPS BY LECTIN MICROARRAY
For differential analysis of fibrosis-associated changes, we initially used sera from healthy volunteers having normal liver function and patients with liver cirrhosis or chronic hepatitis. Sera from patients with rheumatoid arthritis were included as inflammation controls. AGPs were enriched from 0.5-µL aliquots of sera by immunoprecipitation through use of a biotinylated anti-AGP monoclonal antibody conjugated with streptavidin-coated magnetic beads. The heating temperature for elution of the captured AGP was fixed at 60°C, since contamination of streptavidin and biotinylated antibody was minimized while maintaining high elution efficiency and reproducible glycan profiles (see Fig. 2 in the online Data Supplement). An aliquot of the eluate containing nanogram quantities of AGP was then subjected to the antibody-overlay lectin microarray (18). We obtained glycan profiles with signal spots for 15 lectins of the 43-lectin array, namely, AAL, AOL, MAL, SNA, SSA, TJA-I, PHA-L4, RCA120, PHA-E4, DSA, ABA, LEI, STL, UDA, and WGA (Fig. 1). The pattern of signals enabled us to distinguish between the cirrhosis group and the noncirrhosis group (chronic hepatitis, normal liver function, and rheumatoid arthritis). In particular, the fucose-binding lectins (Fuc-binders) AOL and AAL preferentially bound to the AGPs of the cirrhosis group compared with those of the noncirrhosis group. These results are consistent with previous reports showing that α-1,3 fucosylation of a lactosamine unit, the Lewis X antigen, is increased in AGP molecules (19, 28).

STATISTICS
We used either SPSS II software for Windows (SPSS) or Origin version 7.5 software for Windows (OriginLab) to perform the statistical analysis of each lectin signal. To estimate the correlation of lectin signals with liver fibrosis, the relative intensities of lectins in samples from 125 patients, and their platelet counts, were plotted as box plots that described the median and interquartile range. We estimated the abilities of lectins to monitor fibrosis progression from P values calculated by a nonparametric test (the Mann–Whitney U-test). To assess the classification efficiencies of the various lectin signals as markers of significant fibrosis, severe fibrosis, and cirrhosis as described by Leroy et al. (25), receiver operating characteristic (ROC) curve analysis was also done to determine the area under curve (AUC) values. To classify the patients, we used cutoff values obtained from Youden’s index (26). Diagnostic accuracy of each test was expressed as the overall accuracy, diagnostic odds ratio, and AUC (27).
To optimize the detection system for the progression of fibrosis, we next analyzed AGPs from patients with liver cirrhosis or chronic hepatitis (n = 10 for each) with HCV infection. First, we examined the effect of various concentrations of AGP on the glycan profile. The binding signal derived from the lectin microarray analysis of 4 AGP concentrations (there was a 5-fold difference between the highest and lowest concentrations) was normalized by using the “max-normalization” procedure (29). This procedure showed that the glycan profiles of a particular patient were identical, regardless of AGP concentration. A profile based on the signal pattern of 12 selected lectins enabled us to classify the 20 patients into a cirrhosis group or noncirrhosis group. A profile based on the signal pattern of 12 selected lectins enabled us to classify the 20 patients into a cirrhosis group or noncirrhosis group. The classification was done by using a fingerprinting method to classify the 12 lectins. Half of the lectins (DSA, SNA, SSA, TJA-I, WGA, and RCA120) served as internal standards (i.e., as a “pattern formatter”), and the remaining half (AOL, AAL, PHA-E4, LEL, STL, and MAL) served as “fibrosis indicators” (see Fig. 3 in the online Data Supplement). From a technological perspective, the assay offers the following advantages: (a) eluate is directly injected onto the array glass slide without the need for a protein quantification process such as Western blotting or an ELISA, and (b) reliable classification is achieved by using multiplex lectins (i.e., it is error tolerable).

To estimate the reproducibility of the lectin microarray analysis, 45 immunoprecipitated preparations from serum of a particular healthy volunteer were subjected to the lectin microarray analysis. We obtained signal intensities of 10 lectins (i.e., MAL, SNA, SSA, TJA-I, RCA120, DSA, PHA-E4, LEL, STL, and WGA) within the dynamic range (from 1000 to 40 000). When each lectin signal was normalized by the signal of DSA, the CVs of 9 lectins ranged from 6.3% to 14.3% (mean CV 10.5%). Next, we examined the day-to-day variation. For this purpose, we enriched AGPs from 50-μL aliquots of sera. Seven wells of the array glass slide were used for the analysis per day, and the analysis was continued for 9 days. As shown in Fig. 4 in the online Data Supplement, the relative intensity of each lectin was acquired with small day-to-day variation.

**SELECTION OF BEST LECTIN SET FOR MONITORING FIBROSIS PROGRESSION**

To confirm that the signal pattern of the lectin microarray correlated with the progression of fibrosis, we analyzed the AGPs of 125 HCV patients. Fibrosis was graded according to the Histologic Activity Index by using biopsies or surgical specimens. The distribution of fibrosis stage in these patients was as follows: F0–F1, 26.4% (33 patients); F2, 25.6% (32 patients); F3, 24% (30 patients); and F4, 23.2% (29 patients). All patients with fibrosis stage 4 (F4) in this study had Child-Pugh class A cirrhosis. After normalizing the lectin signal data to the signal of an internal standard lectin (DSA), we used the Mann–Whitney U-test as a nonparametric test (P value) to identify the most reliable fibrosis indicator. As shown in Table 2, the P values of all lectins except STL were <0.0001, indicating that they were strongly correlated with liver fibrosis. Among them, we selected AOL as the best fibrosis indicator. As shown in Table 2 and Fig. 2, AOL values were exponentially in-
creased with the progression of fibrosis and were significantly \((P = 1.9 \times 10^{-13})\) higher in patients with severe fibrosis (F3 and F4). In particular, a significant difference was observed between F2 and F3 \((P < 0.001)\). We found that MAL was also one of the candidate indicators. MAL values gradually decreased with the fibrosis progression and were significantly lower in patients with cirrhosis (F4) \((P = 1.5 \times 10^{-13})\). Incidentally, we did not detect a correlation between the intensity of necroinflammatory lesions and the lectin signals (see Fig. 5 in the online Data Supplement).

We next examined AUC to characterize the diagnostic accuracy of each lectin value at each stage of fibrosis, i.e., significant fibrosis (F2/F3/F4), severe fibrosis (F3/F4), and cirrhosis (F4). For the prediction of significant fibrosis, AUC, diagnostic sensitivity, and diagnostic specificity of AOL were 0.76%, 62%, and 88%, respectively (Fig. 3). These values were close to the values of the tests using the glyco-alteration in serum total IgG and specific IgG against \(\alpha\)-Gal epitope, which are known to be high-performance biomarkers for liver fibrosis \((30, 31)\). AOL preferentially distinguished be-

### Table 2. Selection of lectins as fibrosis indicators.

<table>
<thead>
<tr>
<th>n = 125</th>
<th>Significant fibrosis (F2–F4)</th>
<th>Severe fibrosis (F3–F4)</th>
<th>Cirrhosis (F4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative intensity (%)*</td>
<td>Relative intensity (%)*</td>
<td>Relative intensity (%)*</td>
<td></td>
</tr>
<tr>
<td>Lectin</td>
<td>F0–F1 (n = 33)</td>
<td>F2–F4 (n = 92)</td>
<td>U-test (P)</td>
</tr>
<tr>
<td></td>
<td>Relative intensity (%)</td>
<td>Relative intensity (%)</td>
<td>Relative intensity (%)</td>
</tr>
<tr>
<td></td>
<td>U-test (P)</td>
<td>U-test (P)</td>
<td>U-test (P)</td>
</tr>
<tr>
<td>AOL</td>
<td>1.8 (2.0)</td>
<td>13.4 (17.7)</td>
<td>7.2E–06</td>
</tr>
<tr>
<td>MAL</td>
<td>16.9 (4.1)</td>
<td>11.4 (6.8)</td>
<td>6.9E–05</td>
</tr>
<tr>
<td>PHA-E4</td>
<td>28.5 (3.8)</td>
<td>21.9 (8.0)</td>
<td>2.6E–05</td>
</tr>
<tr>
<td>AAL</td>
<td>16.8 (8.2)</td>
<td>33.3 (22.2)</td>
<td>3.5E–05</td>
</tr>
<tr>
<td>LEL</td>
<td>85.1 (10.8)</td>
<td>68.9 (25.6)</td>
<td>3.7E–04</td>
</tr>
<tr>
<td>STI</td>
<td>39.9 (6.3)</td>
<td>36.1 (9.1)</td>
<td>3.4E–02</td>
</tr>
</tbody>
</table>

Data are means (SD) unless otherwise indicated.

Fig. 2. Correlation of lectin signals with liver fibrosis.

Box and whisker plots show the mean (X), the 75th and 25th percentiles (the top and bottom of the box, respectively), the median (line through the middle of the box), and the 95th and 5th percentiles (the top and bottom of the whiskers, respectively). 

\(P\) value was calculated with a nonparametric test (the Mann–Whitney U-test) (bottom).
between F2 and F3. In fact, diagnosis parameters for severe fibrosis were an AUC of 0.85, a diagnostic sensitivity of 78%, and a diagnostic specificity of 78%. On the other hand, MAL could detect cirrhosis with an AUC of 0.91, a diagnostic sensitivity of 93%, a diagnostic specificity of 76%, and a diagnostic odds ratio of 42, which was better than AOL (AUC 0.87, diagnostic sensitivity 83%, and diagnostic specificity 85% for cirrhosis).

Additionally, we analyzed a series of sera from 2 patients with fibrosis stage F4 (blood specimens of these HCV patients were collected at 0.5-year or 1.0-year intervals). This retrospective analysis showed that signal changes gradually occurred in AOL and MAL, accompanied by the progression of fibrosis, whereas the platelet count had no correlation with the progression of fibrosis (see Fig. 6 in the online Data Supplement). It is noteworthy that the signal changes for both AOL and MAL in the HCC patient with advanced fibrosis invariably preceded the corresponding changes in the patient with liver cirrhosis. These results suggest that a test based on the ratio of the AOL or MAL signal to the DSA signal on the lectin microarray would enable the progression of liver fibrosis, as well as the development of cirrhosis, to be monitored.

**APPLICATION OF LECTIN SIGNALS ON LECTIN MICROARRAY TO DIAGNOSE LIVER CIRRHOSIS**

We validated the relative intensity of AOL and MAL signals normalized by DSA signal (AOL/DSA and MAL/DSA). To establish a cutoff value for each signal, we used a data set of 125 patients mentioned previously. We compiled ROC curves to estimate diagnostic sensitivity, diagnostic specificity, and cutoff values (8.0% for AOL/DSA and 11.8% for MAL/DSA; Table 3). Through use of these cutoff values, we tested the accuracy of the immunoassay for the diagnosis of liver cirrhosis by using a validation data set comprised of another 88 patients (liver cirrhosis, n = 45; chronic hepatitis, n = 43) in a single-blind trial. AOL/DSA and MAL/DSA detected liver cirrhosis with 86% (37/43) and 91% (41/45) diagnostic sensitivity, 91% (39/43) and 89% (40/5) diagnostic specificity, and 89% (78/88) and 90% (79/88) overall accuracy, respectively (Table 3 and Table 1 in the online Data Supplement). However, the likelihood ratios (LRs) of a positive test result (LR+), defined as the ratio of a positive test result among liver cirrhosis patients to the same result in chronic hepatitis patients, i.e., sensitivity/(1 − specificity), and a negative test result (LR−), defined as ratio of a negative test result among liver cirrhosis patients to the same result in chronic hepatitis patients, i.e., (1 − sensitivity)/specificity, were not achieved to >10 and <0.1 in the both cases (27). To enhance the diagnostic accuracy by combining both the lectin signals, we developed the following:

\[
L_{\text{Comb}} = \frac{(\text{NI}_{\text{AOL}} \times 1.5 - \text{NI}_{\text{MAL}})}{\text{NI}_{\text{DSA}}} \tag{1}
\]

where NI is net intensity.

Each value \(L_{\text{Comb}}\) given in equation 1 was subsequently used for ROC analysis. As shown in Table 3, the
AUC for \( I_{Comb} \) was 0.90, which was midway between that for MAL/DSA (0.91) and AOL/DSA (0.87). By using the most efficient cutoff value \((-2\)\), we were able to distinguish between liver cirrhosis and non–liver cirrhosis patients with 95% (41/43) diagnostic sensitivity, 91% (41/45) diagnostic specificity, and 93% (82/88) accuracy (Table 3 and Table 1 in the online Data Supplement). Notably, LR+ accuracy (Table 3 and Table 1 in the online Data Supplement). We statistically evaluated the relationships between each lectin signal and the progression of fibrosis diagnosed histopathologically. The analysis identified the most useful indicator lectins for the evaluation of the fibrosis progression. In the subsequent blind test, we confirmed that the combinational use of 3 lectin signals allowed us to identify F4 patients as having liver cirrhosis. The diagnostic accuracy was greatly improved compared with a previous report (20). There remains construction of a rapid and simple sandwich ELISA kit with the lectin set we optimized in this study, before the next validation phase toward a much larger cohort. The optimized assay could be compared with other recently introduced glyco-based test approaches by using the same sample set (30, 31).

An additional key point of our system is the ability to quantify the lectin signals, which would greatly improve the reliability of the assay and enable dynamic monitoring of patients. The developed assay system allowed us to carry out a retrospective analysis of a series of patient sera to evaluate the correlation between changes in the indicator lectin signals (biochemical parameter) and the progression of liver fibrosis (clinical parameter; see Fig. 6 in the online Data Supplement). The proposed monitoring method might facilitate classification of chronic hepatitis patients with respect to disease progression and thereby enable intensive surveillance and follow-up of the chronic hepatitis patients to estimate their risk of developing HCC. The current standard treatment, pegylated interferon-\(\alpha\) plus ribavirin, does not provide sustained virologic response in all patients with HCV. Two SNPs near the gene \( IL28B \) on chromosome 19 are associated with null virologic response in the treatment of patients with HCV genotype 1 (32). Studies on new antiviral agents and treatment approaches are required. Our study is the first to apply a lectin array–based sandwich immunoassay system to the clinical evaluation of glyco-alteration of AGP enriched from 0.5 \( \mu \)L serum in the verification phase (step 1 in Fig. 1 in the online Data Supplement). We statistically evaluated the relationships between each lectin signal and the progression of fibrosis diagnosed histopathologically. The analysis identified the most useful indicator lectins for the evaluation of the fibrosis progression. In the subsequent blind test, we confirmed that the combinational use of 3 lectin signals allowed us to identify F4 patients as having liver cirrhosis. The diagnostic accuracy was greatly improved compared with a previous report (20). There remains construction of a rapid and simple sandwich ELISA kit with the lectin set we optimized in this study, before the next validation phase toward a much larger cohort. The optimized assay could be compared with other recently introduced glyco-based test approaches by using the same sample set (30, 31).

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biomarkers. Because the glycosylation features strongly depend on tissue origin and the presence of malignancy, blood glycoproteins consist of a considerable mixture of heterogeneous molecules derived from many sources, although their protein moieties are the same. Thus, detection of serum glycoproteins exhibiting cancer-associated glyco-alteration can be extremely useful in biomarker investigation (33). Until now, however, there was no reliable assay targeting the glycoprotein biomarkers. The absence of a “rational” system for verification of glyco-biomarker candidates and selection of the most efficient lectin was a major impediment to the development of a reliable sandwich immunoassay. In the present study, we applied an antibody-assisted lectin profiling system to identify the most useful lectin for the validation of fibrosis marker AGP. The glyco-alteration of AGP was completely depicted by using the most reliable lectin set, which was rationally identified by using the lectin array–based sandwich immunoassay before the step 3 in Fig. 1 in the online Data Supplement. These processes should contribute to reducing the current labor of the time-consuming glyco-marker development and thus expedite future innovation in the field of biomarkers.

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