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Hepatitis C virus kinetics by administration of pegylated interferon-α in human and chimeric mice carrying human hepatocytes with variants of the *IL28B* gene

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Comparison of LecT-Hepa and FibroScan for assessment of liver fibrosis in hepatitis B virus infected patients with different ALT levels

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ABSTRACT

Background: FibroScan is one of the noninvasive techniques based on the transient elastography that can assess the progression of liver fibrosis in chronic hepatitis patients in daily clinical practice. Recently, LecT-Hepa was validated as a serological glycomarker correlating well with the fibrosis stage determined by liver biopsy, and was superior to many other noninvasive biochemical markers and tests. We compared the reliability of LecT-Hepa with that of FibroScan for evaluation of liver fibrosis.
Methods: The effects of increased alanine aminotransferase (ALT) activities on LecT-Hepa and FibroScan were investigated.
Results: The areas under the receiver-operating characteristic curves, sensitivity and specificity for detecting cirrhosis, which is one of the outcomes of fibrosis estimation, were 0.82, 72.5% and 78.2% of LecT-Hepa, 0.85, 87.0% and 74.1% of FibroScan; these did not differ significantly. The count distribution of LecT-Hepa in non-cirrhosis group or cirrhosis group did not differ between the patients grouped according to their ALT levels, whereas that of FibroScan was substantially affected.
Conclusion: LecT-Hepa was confirmed as a reliable noninvasive test for the evaluation of liver fibrosis in hepatitis B virus-infected patients with comparable performance to that of FibroScan and proved to be unaffected by inflammation.

1. Introduction

It is estimated that about 2 billion people worldwide have been infected with the hepatitis B virus (HBV), and >350 million of them have chronic HBV infection [1]. In China, a seroepidemiological survey of HBV infection in 2006 showed that the prevalence of hepatitis B surface antigen positivity was 7.18%. It was estimated that 93 million people were HBV carriers, of whom 30 million were patients with chronic hepatitis B (CHB) [2]. CHB may progress to cirrhosis and hepatocellular carcinoma. An accurate method for monitoring the progression of liver fibrosis is urgently needed for the prognosis and management of chronic liver diseases. Liver biopsy is generally considered as the gold standard for assessing hepatic histology in CHB [3–5]. However, it often has limitations due to its invasiveness, risk of complications, sampling errors, and interobserver variability [6–8]. Many noninvasive methods for replacing or complementing the liver biopsy have been developed in recent years [9–12]. FibroScan (transient elastography) and FibroTest (serological marker test) have been evaluated most frequently; these methods have similar diagnostic accuracies for predicting fibrosis staging from receiver-operating characteristic (ROC) curves [13–16]. FibroTest employs a narrow and complex algorithm for 5 biochemical markers (α2-macroglobulin, apolipoprotein A1, haptoglobin, γ-glutamyl transferase, and bilirubin), which requires extensive and specialized blood analysis [17]. Recently, we developed a novel diagnostic score named LecT-Hepa for convenient and rapid monitoring of liver fibrosis progression. It is based on glyco-alteration (e.g., fucosylation and desialylation) of serum α1-acid glycoprotein

Abbreviations: HBV, hepatitis B virus; CHB, chronic hepatitis B; PLT, platelet count; AGP, α1-acid glycoprotein; LSM, liver stiffness measurement; LC, liver cirrhosis; non-LC, non-cirrhosis; DSA, *Datura stramonium* agglutinin; MAL, *Maackia amurensis* lectin; AOL, *Aspergillus oryzae* lectin.

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(AGP), which is assessed using a triplex lectin–antibody immunoassay [18,19]. It has been demonstrated to be well correlated with the fibrosis stage determined by liver biopsy, and verified to be more efficient by comparing with other serological methods (hyaluronic acid, tissue inhibitor of metalloproteases-1, platelet count, APRI, Forns index, Fib-4 index, and Zeng's score) in a multicenter study [20]. Here, to evaluate the reliability of LecT-Hepa for assessing liver fibrosis, we compared the diagnostic performance of LecT-Hepa and FibroScan for distinguishing cirrhosis from non-cirrhosis in a large cohort of HBV-infected Chinese patients with different serum alanine aminotransferase (ALT) levels.

2. Materials and methods

2.1. Patients

A total of 239 patients who had been positive for hepatitis B surface antigen for at least 6 months were enrolled retrospectively from Ruijin Hospital (Shanghai, China) from March 2009 to May 2011. Patients who were coinfected with another hepatitis virus or HIV, or who had excessive alcohol intake (>20 g/d), hepatocellular carcinoma, or other causes of liver diseases were excluded. For all patients, serum biochemical parameters, including the levels of aspartate aminotransferase (AST) and ALT, as well as platelet (PLT), were assessed at the time of the liver stiffness measurement. Normal values for ALT and AST ranged between 10 and 64 IU/l and between 8 and 40 IU/l, respectively, which were determined based on the manufacturer's instructions and adjusted according to the results of validation test by medical laboratory of Ruijin Hospital. Serum samples were collected at the time of the liver stiffness measurement for detection of lectins and stored at −20 °C until analysis. The patients were divided into two groups: liver cirrhosis (LC) group and non-cirrhosis (non-LC) group. The diagnosis of cirrhosis was based on clinical and morphological criteria and ultrasonography according to standard definitions [21]. The institutional ethics committees of Ruijin Hospital of Shanghai Jiao Tong University approved this study, and the informed consent was obtained from all patients.

2.2. Liver stiffness measurement

Liver stiffness was measured by transient elastography using FibroScan (EchoSens, Paris, France). The measurement depth was between 25 mm and 65 mm. For each patient, 10 validated measurements were performed. The success rate was calculated as the number of validated measurements divided by the total number of measurements. The results were expressed in kilopascals. The median value was considered representative of the elastic modulus of the liver. Only procedures with 10 validated measurements and a success rate of at least 60% were considered reliable.

2.3. Automatic acquisition of quantitative glyco-alteration of AGP

Each individual serum (5 μl) that had been stored at −20 °C was diluted 10 fold with phosphate buffered saline containing 0.2% sodium dodecyl sulfate, and then heated at 95 °C for 20 min. AGP in the resulting solution was enriched by immunoprecipitation with biotinylated anti-AGP antibody using an automated protein purification system (ED-01; GP BioSciences Ltd., Tokyo, Japan). Each elution fraction (100 μl) was kept at −80 °C until a sandwich immunoassay was performed. Subsequent to the enrichment, fibrosis-specific glyco-alteration of AGP was quantified using simultaneous lectin–antibody sandwich immunoassays for three lectins: *Datura stramonium* agglutinin (DSA), *Maackia amurensis* lectin (MAL), and *Aspergillus oryzae* lectin (AOL), by a fully automatic chemiluminescence enzyme immunoassay system (HISCL-2000i; Sysmex Co., Kobe, Japan). The criterion formula of LecT-Hepa was as before described [19]:

$$\text{LecT-Hepa} = \text{Log}_{10}[\text{AOL/DSA}] \times 8.6 - [\text{MAL/DSA}].$$

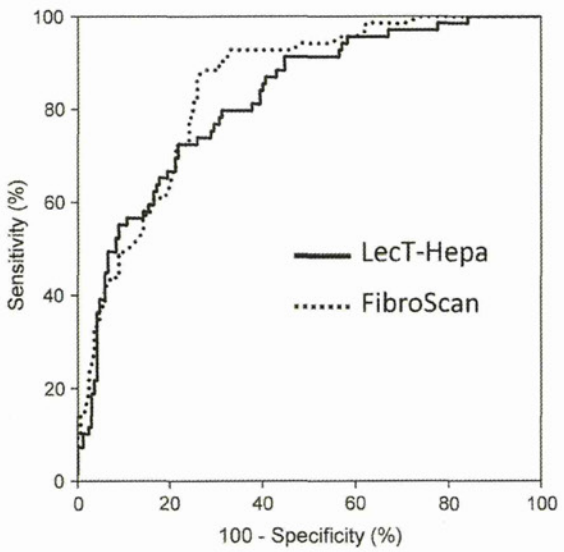
2.4. Statistical analysis

Statistical calculations were performed using software from GraphPad Prism 5 (GraphPad, San Diego, CA). A P value of <0.01 (1%) was considered to be statistically significant. The diagnostic performance of the fibrosis markers and indices were assessed using ROC curves and were then expressed as diagnostic specificity, sensitivity,

Table 1
Clinical characteristics of the patients.

Data	non-LC ($n=170$)	LC ($n=69$)	Significance non-LC vs LC
Age (y)	38.5 ± 11.0	47.94 ± 9.0	$P<0.0001$
Male sex (%)	126 (74.1%)	51 (73.9%)	–
AST (IU/l)	70.5 ± 150.1	88.4 ± 109.8	$P=0.0002$
ALT (IU/l)	111.6 ± 213.7	88.5 ± 116.1	$P=0.1965$
PLT ($\times 10^9$/l)	167.5 ± 43.9	86.0 ± 48.0	$P<0.0001$
FibroScan	10.3 ± 8.8	27.0 ± 19.1	$P<0.0001$
MAL/DSA	10.1 ± 2.0	7.5 ± 2.3	$P<0.0001$
AOL/DSA	5.1 ± 13.5	24.0 ± 47.6	$P<0.0001$

Patients were classified as non-LC or LC. LC, liver cirrhosis; non-LC, non-cirrhosis. Quantitative results are expressed as means ± standard deviations or n (%).



	FibroScan	LecT-Hepa
AUC	0.85	0.82
(95% CI)	(0.797-0.897)	(0.763-0.877)
Se (%)	87.0	72.5
Sp (%)	74.1	78.2
PPV (%)	57.7	57.5
NPV (%)	93.3	87.5

Fig. 1. Receiver-operating characteristic curves of LecT-Hepa and FibroScan for distinguishing LC from non-LC. AUC, area under the receiver-operating characteristic curve; Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value.

positive predictive value (PPV), negative predictive value (NPV) and area under the ROC curve (AUC) values (95% confidence interval [95% CI]).

3. Results

3.1. General characteristics

A total of 239 patients who showed evidence of chronic HBV infection and had undergone liver stiffness measurement were investigated. The mean age was 41.2 ± 11.3 y, and 177 (74%) of them were males. Among the all, 170 (71%) and 69 (29%) patients were diagnosed as non-LC and LC, respectively. Their characteristics are summarized in Table 1. Significant differences were found in Age ($P<0.0001$), AST ($P=0.0002$), PLT ($P<0.0001$), FiborScan ($P<0.0001$), MAL/DSA ($P<0.0001$), and AOL/DSA ($P<0.0001$) between the non-LC group and LC group, whereas ALT ($P=0.1965$) was not significantly different between the two groups.

3.2. Receiver-operating characteristic analysis

The overall diagnosis performances of LecT-Hepa and FibroScan were assessed using ROC curves. Fig. 1 shows the ROC curves for distinguishing LC from non-LC by both methods. The area under the ROC curve (95% CI) was 0.82 (0.763–0.877) for LecT-Hepa and 0.85 (0.797–0.897) for FibroScan. The overall diagnostic accuracies for LecT-Hepa and FibroScan were 77% and 78%, respectively. The obtained values for sensitivity, specificity, PPV, and NPV are shown in the bottom table of Fig. 1. There was no significant difference between both methods.

3.3. Effect of hepatic inflammation on the diagnostic cutoff values

Because the upper limit of the normal value for ALT level was 64 IU/l, the patients were categorized by the normal (≤64 IU/l) and elevated (>64 IU/l) ALT levels. According to this classification, 169 patients (71%) had the normal ALT level and 70 patients (29%) had the elevated ALT level. The proportions of patients with LC in the normal and elevated ALT levels were similar (28% of normal ALT patients and 30% of elevated ALT patients). Distribution of the values obtained by each test is shown in Fig. 2. Medians of these methods increased significantly between the non-LC group and LC group (all $P<0.0001$) in the both ALT levels. LecT-Hepa values in the non-LC group ($P=0.65$) and LC group ($P=0.02$) showed no significant difference between the two ALT categories (Fig. 2A). In contrast, the FibroScan value was obviously increased with the elevation of ALT levels ($P<0.0001$) even in the same diagnostic group (Fig. 2B). Thereby, we could distinguish the LC group in the normal ALT level from non-LC group in the elevated ALT level ($P<0.0001$) by LecT-Hepa, but could not by FibroScan ($P=0.05$). Collectively, the value of FibroScan was greatly affected by the ALT levels, whereas the value for LecT-Hepa was not influenced regardless of the ALT levels.

4. Discussion

This is the first study comparing LecT-Hepa with FibroScan. These results showed the obvious advantage of LecT-Hepa in comparison with FibroScan based on robustness against fluctuation of the ALT levels with a large cohort of HBV-infected Chinese patients at different ALT levels. Thus, the diagnostic performance of LecT-Hepa was the most reliable for monitoring the progression of hepatic fibrosis.

A recent paper showed that the majority of nucleoside-naïve patients with CHB who were treated with entecavir in the long-term cohort achieved substantial histological improvement and regression of fibrosis or cirrhosis [22], suggesting that a noninvasive test for the assessment of liver fibrosis in the treated patients is required during the follow-up. The liver biopsy is limited not only by its invasive nature, but also by its accuracy. A specimen collected in a standard liver biopsy using a short, narrow-gauge needle represents a very small portion of the whole liver mass, resulting in intra- and interobserver variability and sampling errors, which account for 25% of false-negative diagnoses of cirrhosis [23–25]. Therefore, a noninvasive marker that accurately reflects the condition of the whole liver is required.

At present, FibroScan is the most intensively evaluated noninvasive method for the assessment of liver fibrosis. Its diagnostic value is considered to be superior to that of biochemical markers [26]. However, several studies noted that liver stiffness measurements using FibroScan for patients with inflammation and acute liver damage overestimate the actual stage of fibrosis and may reduce the diagnostic accuracy [27,28]. In general, a high ALT level reflects a vigorous immune response

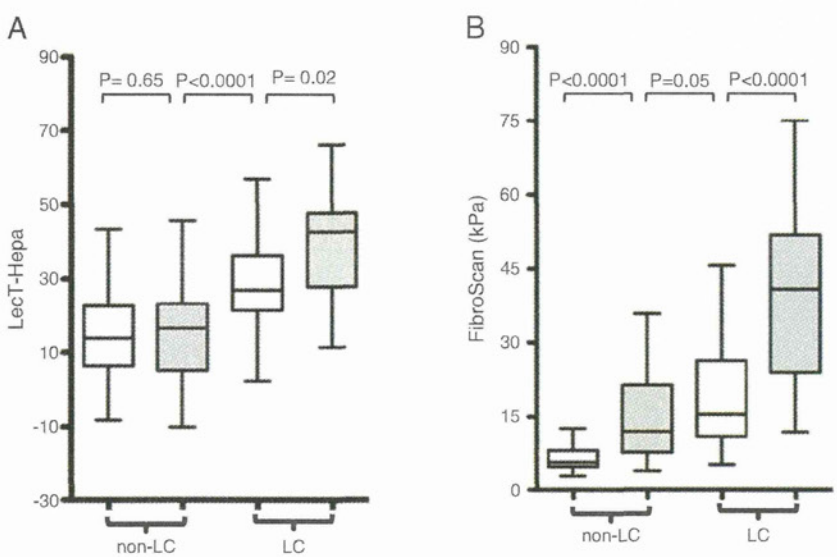


Fig. 2. Distribution of LecT-Hepa (A) and FibroScan (B) values in non-LC and LC patients with different ALT levels. The top and bottom of the whiskers are the 95th and 5th percentiles. The top and bottom of the boxes are the first and third quartiles. The size of the box represents the interquartile range within which 50% of the values are located. The line across the box indicates the median value. LC, liver cirrhosis; non-LC, non-cirrhosis. The open and gray boxes indicate normal (≤64 IU/l) and elevated (>64 IU/l) ALT levels, respectively.

to HBV and histological activity (i.e., necroinflammation). Our study obviously showed that the FibroScan values were substantially affected by ALT fluctuation. These results were also in accordance with the study of Kim et al., in which advanced fibrosis stage (F3–4) or cirrhosis showed a negative correlation with discordance between liver biopsy and FiborScan in assessing liver fibrosis in patients with CHB, and maximal activity grade 3–4 significantly influenced the liver stiffness measurement values in F3 and F4 [28]. In practice, hepatic activation and fibrosis stage should be estimated independently, as should histological diagnoses followed by a biopsy, such as the histological activity index scoring system. Thus, a marker that relies on an analysis of the specific protein content to monitor liver fibrosis should be robust against hepatic inflammation. In this context, we can explain that the reliability of LecT-Hepa is superior to that of FibroScan. LecT-Hepa has been already validated for estimating liver fibrosis using a large amount of serum specimens from patients with well-defined fibrosis stage by biopsy in a multicenter study [21]. This report led us to consider that LecT-Hepa can be a good substitute for liver biopsy. This is the reason we herein focused on the examination into the effect of hepatic inflammation on diagnosis of LC by LecT-Hepa.

In conclusion, we confirmed that LecT-Hepa is unaffected by inflammation. This suggested that LecT-Hepa is the most reliable and effective for the assessment of fibrosis progression in HBV-infected patients whose ALT levels are often fluctuated and thus can be used for routine assessments of liver fibrosis in HBV-infected patients.

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LecT-Hepa, a Glyco-Marker Derived from Multiple Lectins, as a Predictor of Liver Fibrosis in Chronic Hepatitis C Patients

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Assessment of liver fibrosis in patients with chronic hepatitis C (CHC) is critical for predicting disease progression and determining future antiviral therapy. LecT-Hepa, a new glyco-marker derived from fibrosis-related glyco-alteration of serum alpha 1-acid glycoprotein, was used to differentiate cirrhosis from chronic hepatitis in a single-center study. Herein, we aimed to validate this new glyco-marker for estimating liver fibrosis in a multicenter study. Overall, 183 CHC patients were recruited from 5 liver centers. The parameters *Aspergillus oryzae* lectin (AOL) / *Dature stramonium* lectin (DSA) and *Maackia amurensis* lectin (MAL)/DSA were measured using a bedside clinical chemistry analyzer in order to calculate LecT-Hepa levels. The data were compared with those of seven other noninvasive biochemical markers and tests (hyaluronic acid, tissue inhibitor of metalloproteases-1, platelet count, aspartate aminotransferase-to-platelet ratio index [APRI], Forns index, Fib-4 index, and Zeng's score) for assessing liver fibrosis using the receiver-operating characteristic curve. LecT-Hepa correlated well with the fibrosis stage as determined by liver biopsy. The area under the curve (AUC), sensitivity, and specificity of LecT-Hepa were 0.802, 59.6%, and 89.9%, respectively, for significant fibrosis; 0.882, 83.3%, and 80.0%, respectively, for severe fibrosis; and 0.929, 84.6%, and 88.5%, respectively, for cirrhosis. AUC scores of LecT-Hepa at each fibrosis stage were greater than those of the seven aforementioned noninvasive tests and markers. *Conclusion*: The efficacy of LecT-Hepa, a glyco-marker developed using glycoproteomics, for estimating liver fibrosis was demonstrated in a multicenter study. LecT-Hepa given by a combination of the two glyco-parameters is a reliable method for determining the fibrosis stage and is a potential substitute for liver biopsy. (HEPATOLOGY 2012;56:1448-1456)

Accurate staging of hepatic fibrosis in patients with chronic hepatitis C (CHC) is most important for predicting disease progression and determining the need for initiating antiviral therapy, such as interferon (IFN) therapy.[1,2] Liver biopsy has been considered the gold standard for fibrosis staging for many years.[3] However, liver biopsy is invasive and painful,[4,5] with rare but potentially life-threatening complications.[6] In addition, this method may suffer from sampling errors since only 1/50,000 of the organ is examined.[7] Furthermore, inter- and intraobserver discrepancies reaching levels of 10% to 20% have been

Abbreviations: α2-MG, α2-macroglobulin; AFP, alpha-fetoprotein; AGP, alpha-1 acid glycoprotein; ALT, alanine aminotransferase; AOL, Aspergillus oryzae *lectin; CHC, chronic hepatitis C; DSA,* Datura stramonium *lectin; GGT, gamma-glutamyltransferase; HA, hyaluronic acid; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; MAL,* Maackia amurensis *lectin; TIMP1, tissue inhibitors of metalloproteinases 1.*

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reported using this method, leading to misdiagnosis of cirrhosis.[8] Therefore, finding a noninvasive method for diagnosing liver fibrosis is an emerging issue in the care of patients with CHC.

Several methods have been studied for the noninvasive diagnosis of hepatic fibrosis or cirrhosis, including clinical[9] or blood markers,[10,11] and signal analysis (ultrasonography, magnetic resonance imaging, and elastography).[12,13] Although each method can play a substantial role in the diagnosis of cirrhosis, it is evident that the best way of monitoring hepatitis progression employs an accurate serological method for the quantitative evaluation of fibrosis. We developed a new glyco-marker using multiple lectins that performed well in estimating liver fibrosis in a single-center study.[14,15]

Recent progress in glycoproteomics has had a great influence on work toward ideal, disease-specific biomarkers for a number of conditions. Glycoproteins that exhibit disease-associated glyco-alteration and are present in serum or other fluids have the potential to act as biomarkers for the diagnosis of a target disease,[16] because the features of glycosylation depend on the extent of cell differentiation and the stage of the cell. Detecting hepatic disease-associated glyco-markers for clinical applications has been a continuous challenge since the early 1990s, because increased fucosylation on complex-type *N*-glycans has been frequently detected in glycoproteins from patients with hepatocellular carcinoma (HCC) and cirrhosis.[17,18] Of all the alpha-fetoprotein (AFP) glycoforms, more than 30% have been found to react to a fucose-binding lectin, *Lens culinaris* agglutinin. This fraction, designated AFP-L3, was approved by the U.S. Food and Drug Administration (FDA) in 2005 for the diagnosis and prognosis of HCC.[19] We have found that two fibrosis-indicator lectins (*Aspergillus oryzae* lectin [AOL] and *Maackia amurensis* lectin [MAL]) together with an internal, standard lectin (*Datura stramonium* lectin [DSA]) on an alpha 1-acid glycoprotein (AGP) could, using lectin microarray, clearly distinguish between cirrhosis and chronic hepatitis patients.[14] We have further simplified this quantitative method so that it could be performed using bedside, clinical chemistry analyzers.[15]

The aim of the current study was to evaluate this new glyco-marker (LecT-Hepa) using multiple lectins and bedside clinical chemistry analyzers for use in the assessment of liver fibrosis. In this multicenter study we compared the method's efficiency in estimating liver fibrosis with other noninvasive fibrosis markers and tests.

Materials and Methods

Study Population. This study included 183 consecutive adult patients with CHC who had undergone percutaneous liver biopsy at one of the following institutions: Hokkaido University Hospital, Musashino Red Cross Hospital, National Center for Global Health and Medicine, Hyogo College of Medicine Hospital, or Nagoya City University Hospital in Japan. A diagnosis of CHC was defined as detectable serum anti-hepatitis C virus (HCV) antibody and HCV-RNA, found using polymerase chain reaction assays, of at least 2 points. Exclusion criteria were coinfection with hepatitis B virus or human immunodeficiency virus (HIV), and other disorders that commonly cause liver diseases. Informed consent was obtained from each patient who participated in the study. This study was conducted in accordance with the provisions of the Declaration of Helsinki and was approved by our Institutional Review Board.

Histological Staging. Ultrasonography-guided liver biopsy was performed according to a standardized protocol. Specimens were fixed, paraffin-embedded, and stained with hematoxylin-eosin and Masson's trichrome. A minimum of six portal tracts in the specimen were required for diagnosis. All liver biopsy samples were independently evaluated by two senior pathologists who were blinded to the clinical data. Liver fibrosis stages were assessed using METAVIR fibrosis (F) staging.[20] Significant fibrosis was defined as METAVIR F $\geq$2, severe fibrosis as METAVIR F $\geq$3, and cirrhosis as METAVIR F4. Two patients were excluded from the study because of inadequate histological samples.

Clinical and Biological Data. The age and sex of the patients were recorded. Serum samples were collected immediately before or no more than 2 months

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after liver biopsy and were stored at −80°C until analysis. The concentrations of the following variables were obtained by analyzing the serum samples: aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltransferase (GGT), total bilirubin, albumin, cholinesterase, total cholesterol, platelet count (platelets), prothrombin time, haptoglobin, hyaluronic acid (HA), α2-macroglobulin (α2-MG), tissue inhibitors of metalloproteinases 1 (TIMP1). The aspartate aminotransferase-to-platelet ratio index (APRI), Fib-4 index, Forns index, and Zeng's score were calculated according to published formulae appropriate to each measure.[2,7,21,22]

Rapid Lectin-Antibody Sandwich Immunoassay Using HISCL. Fibrosis-specific glyco-alteration of AGP was qualified from simultaneous measurements of the lectin-antibody sandwich immunoassays using three lectins (DSA, MAL, and AOL). In principle, the glycan part of the AGP was captured by the lectin immobilized on the magnetic beads, and the captured AGP was then quantified by an antihuman AGP mouse monoclonal antibody probe that was cross-linked to an alkaline phosphatase (ALP-αAGP). The assay manipulation was fully automated using a chemiluminescence enzyme immunoassay machine (HISCL-2000i; Sysmex, Kobe, Japan). We used the following criterion formula, named the "LecT-Hepa Test," to enhance the diagnostic accuracy by combining two glyco-parameters (AOL/DSA and MAL/DSA) as described before: $F = \mathrm{Log}_{10}[\mathrm{AOL/DSA}]*8.6-[\mathrm{MAL/DSA}]$.[15]

Statistical Analyses. Quantitative variables were expressed as the mean ± standard deviation (SD) unless otherwise specified. Categorical variables were compared using a chi-squared test or Fisher's exact test, as appropriate, and continuous variables were compared using the Mann-Whitney U test. $P < 0.05$ was considered statistically significant. A multivariate forward stepwise logistic regression analysis was performed to determine the independent predictors of the absence or presence of significant fibrosis, severe fibrosis, and cirrhosis, respectively. Pearson's correlation coefficient was used as necessary. To assess the classification efficiencies of various markers for detecting significant fibrosis, severe fibrosis, and cirrhosis,[23] and to determine area under the curve (AUC) values, receiver-operating characteristic (ROC) curve analysis was also carried out. Diagnostic accuracy was expressed as the diagnostic specificity (specificity), diagnostic sensitivity (sensitivity), positive predictive values (PPV), negative predictive values (NPV), positive likelihood ratio (LR [+]), negative likelihood ratio (LR [−]), and AUC (95% confidence interval [95% CI]). We performed statistical analyses using STATA v. 11.0 (StataCorp, College Station, TX).

Table 1. Baseline Characteristics of the 183 Patients with Chronic Hepatitis C at the Time of Liver Biopsy

Features	Total (n = 183)
Age (years)	57.6 ± 11.4
Male sex	75 (41.0)
AST (IU/L)	57.4 ± 43.9
ALT (IU/L)	62.8 ± 56.8
GGT (IU/L)	51.1 ± 62.6
Bilirubin (mg/dL)	0.7 ± 0.4
Albumin (g/L)	4.1 ± 0.4
Cholinesterase (IU/L)	283.5 ± 97.0
Cholesterol (mg/dL)	174.1 ± 35.5
Platelets (10^9/L)	163 ± 57
Prothrombin time (%)	87.2 ± 33.4
α2-MG (g/L)	356.8 ± 133.1
HA (μg/L)	205.3 ± 428.0
TIMP1 (pg/ml)	210.6 ± 87.7
AOL/DSA	6.3 ± 12.3
MAL/DSA	9.0 ± 3.1
Fibrosis stage (%):	
F0-1	89 (48.6)
F2	46 (25.1)
F3	22 (12.0)
F4	26 (14.2)

Results

Baseline Characteristics of the 183 Patients with Chronic Hepatitis C at the Time of Liver Biopsy. Patient characteristics at the time of liver biopsy are shown in Table 1. The mean age of the 183 patients was 57.6 ± 11.4 years, and 75 (41%) of them were men. F0-F1 was diagnosed in 89 cases (48.6%), F2 in 46 (25.1%), F3 in 22 (12.0%), and F4 (cirrhosis) in 26 (14.2%).

Comparison of Variables Associated with the Presence of Significant Fibrosis by Univariate and Multivariate Analysis. Variables associated with the presence of significant fibrosis were assessed by univariate and multivariate analysis (Table 2). The variables of age ($P = 0.001$), AST ($P < 0.0001$), ALT ($P < 0.0001$), GGT ($P < 0.0001$), bilirubin ($P = 0.014$), α2-MG ($P = 0.002$), HA ($P < 0.0001$), TIMP1 ($P < 0.0001$), and AOL/DSA ($P < 0.0001$) were significantly higher in the significant fibrosis group than in the not significant fibrosis group. The variables albumin ($P < 0.001$), cholinesterase ($P < 0.0001$), cholesterol ($P = 0.005$), platelets ($P < 0.0001$), prothrombin time ($P = 0.0001$), and MAL/DSA ($P < 0.0001$) were significantly lower in the significant fibrosis group than in the not significant fibrosis group. Multivariate analysis showed that platelets (odds ratio [OR]: 0.87,

Table 2. Variables Associated with the Presence of Significant Fibrosis (F2-4) and Severe Fibrosis (F3-4) by Univariate and Multivariate Analysis

Features	No Significant Fibrosis (n = 89)	Significant Fibrosis (n = 94)	P Value (Univariate)	Odds Ratio (95% CI) (Multivariate)	No Severe Fibrosis (n = 135)	Severe Fibrosis (n = 48)	P Value	Odds Ratio (95% CI) (Multivariate)
Age (years)	54.7 ± 11.8	60.5 ± 10.4	0.001		55.8 ± 11.9	62.9 ± 7.8	0.001	1.15 (1.02-1.31)
Male sex (%)	30 (33.7)	45 (47.9)	0.051		52 (38.5)	23 (47.9)	0.255	
AST (IU/L)	45.7 ± 41.6	68.3 ± 43.5	<0.0001		49.7 ± 40.1	79.1 ± 47.4	<0.0001	
ALT (IU/L)	51.0 ± 56.6	74.0 ± 54.9	<0.0001		55.9 ± 54.9	82.5 ± 57.9	<0.0001	
GGT (IU/L)	40.6 ± 61.7	62.1 ± 63.1	<0.0001		45.5 ± 67.1	65.8 ± 46.7	<0.0001	
Bilirubin (mg/dL)	0.6 ± 0.3	0.7 ± 0.4	0.014		0.6 ± 0.3	0.8 ± 0.4	0.005	
Albumin (g/L)	4.2 ± 0.3	4.0 ± 0.5	<0.001		4.2 ± 0.3	3.8 ± 0.5	<0.0001	
Cholinesterase (IU/L)	329.2 ± 76.0	247.2 ± 96.9	<0.0001		312.4 ± 84.4	217 ± 91.9	<0.0001	
Cholesterol (mg/dL)	181.0 ± 31.5	167.5 ± 36.2	0.005		178.1 ± 34.1	162.4 ± 33.5	0.016	
Platelets (10^9/L)	186 ± 53	142 ± 52	<0.0001	0.87 (0.77-0.99)	180 ± 52	119 ± 46	<0.0001	0.74 (0.58-0.94)
Prothrombin time (%)	94.7 ± 33.4	80.1 ± 32.1	0.0001		89.5 ± 36.2	80.8 ± 23.2	<0.001	
α2-MG (g/L)	326 ± 117.7	389.2 ± 141.1	0.002		331.1 ± 122.5	423.9 ± 137.5	<0.0001	
HA (μg/L)	85.6 ± 154.3	318.7 ± 556.1	<0.0001	1.01 (1.01-1.02)	115.4 ± 201.1	458.2 ± 711.0	<0.0001	
TIMP1 (pg/ml)	183.5 ± 53.3	238.6 ± 106.1	<0.0001		189.7 ± 64.5	263.9 ± 113.8	<0.0001	
AOL/DSA	1.4 ± 1.2	10.9 ± 15.9	<0.0001	1.51 (1.07-2.15)	2.0 ± 2.6	18.3 ± 19.3	<0.0001	
MAL/DSA	10.6 ± 1.7	7.5 ± 3.4	<0.0001		10.2 ± 2.0	5.6 ± 3.4	<0.0001	0.52 (0.37-0.76)

95% CI: 0.77-0.99), HA (OR: 1.01, 95% CI: 1.01-1.02), and AOL/DSA (OR: 1.51, 95% CI: 1.07-2.15) were independently associated with the presence of significant fibrosis.

Comparison of Variables Associated with the Presence of Severe Fibrosis by Univariate and Multivariate Analysis. Variables associated with the presence of severe fibrosis were assessed by univariate and multivariate analysis (Table 2). The variables of age ($P = 0.001$), AST ($P < 0.0001$), ALT ($P < 0.0001$), GGT ($P < 0.0001$), bilirubin ($P = 0.005$), α2-MG ($P < 0.0001$), HA ($P < 0.0001$), TIMP1 ($P < 0.0001$), and AOL/DSA ($P < 0.0001$) were significantly higher in the severe fibrosis group than in the no severe fibrosis group. The variables albumin ($P < 0.0001$), cholinesterase ($P < 0.0001$), cholesterol ($P = 0.016$), platelets ($P < 0.0001$), prothrombin time ($P < 0.001$), and MAL/DSA ($P < 0.0001$) were significantly lower in the severe fibrosis group than in the no severe fibrosis group. Multivariate analysis showed that age (OR: 1.15, 95% CI: 1.02-1.31), platelets (OR: 0.74, 95% CI: 0.58-0.94), and MAL/DSA (OR: 0.52, 95% CI:

Table 3. Variables Associated with the Presence of Cirrhosis (F4) by Univariate and Multivariate Analysis

Features	No Cirrhosis (n=157)	Cirrhosis (n = 26)	P Value	Odds Ratio (95% CI) (Multivariate)
Age (years)	56.6 ± 11.7	63.8 ± 7.3	0.0016	
Male sex (%)	60 (38.2)	15 (57.7)	0.061	
AST (IU/L)	54.6 ± 41.7	74.9 ± 53.7	0.016	
ALT (IU/L)	62.1 ± 58.1	67.2 ± 48.2	0.446	
GGT (IU/L)	48.5 ± 63.9	64.9 ± 53.8	0.0031	
Bilirubin (mg/dL)	0.6 ± 0.3	1.0 ± 0.5	<0.0001	
Albumin (g/L)	4.2 ± 0.4	3.6 ± 0.5	<0.0001	
Cholinesterase (IU/L)	305.3 ± 83.9	181.7 ± 90.1	<0.0001	
Cholesterol (mg/dL)	178.4 ± 33.3	146.9 ± 29.8	<0.0001	
Platelets (10^9/L)	172 ± 54	106 ± 36	<0.0001	0.76 (0.58-0.99)
Prothrombin time (%)	88.7 ± 35.5	79.2 ± 16.1	0.0004	
α2-MG (g/L)	346.2 ± 131.6	416.9 ± 127.8	0.019	
HA (μg/L)	137.1 ± 215.7	617.4 ± 915.1	<0.0001	
TIMP1 (pg/ml)	196.4 ± 70.4	287.3 ± 126.6	<0.0001	
AOL/DSA	3.4 ± 7.1	24.0 ± 20.4	<0.0001	
MAL/DSA	9.8 ± 2.4	4.2 ± 2.8	<0.0001	0.67 (0.49-0.90)

0.37-0.76) were independently associated with the presence of severe fibrosis.

Comparison of Variables Associated with the Presence of Cirrhosis by Univariate and Multivariate Analysis. Variables associated with the presence of cirrhosis were assessed by univariate and multivariate analysis (Table 3). Age ($P = 0.0016$), AST ($P = 0.016$), GGT ($P = 0.0031$), bilirubin ($P < 0.0001$), α2-MG ($P = 0.019$), HA ($P < 0.0001$), TIMP1 ($P < 0.0001$), and AOL/DSA ($P < 0.0001$) were significantly higher in the cirrhosis group than in the no cirrhosis group. Albumin ($P < 0.0001$), cholinesterase ($P < 0.0001$), cholesterol ($P < 0.0001$), platelets ($P < 0.0001$), prothrombin time ($P = 0.0004$), and MAL/DSA ($P < 0.0001$) were significantly lower in the cirrhosis group than in the no cirrhosis group. Multivariate analysis showed that platelets (OR: 0.76, 95% CI: 0.58-0.99) and MAL/DSA (OR: 0.67, 95% CI: 0.49-0.90) were independently associated with the presence of cirrhosis.

Evaluation of the Two Glyco-Parameters AOL/DSA and MAL/DSA for Estimating the Progression of Liver Fibrosis. To assess the correlation of the two obtained glyco-parameters with the progression of fibrosis, we analyzed the data of triple lectins from HISCL measurements on the 183 CHC patients. The boxplots of AOL/DSA and MAL/DSA in relation to the fibrosis staging are shown in Fig. 1A,B, respectively. The AOL/DSA values gradually increased with the progression of fibrosis and Pearson's correlation efficient was R = 0.61. On the other hand, the MAL/DSA values gradually decreased with the progression of fibrosis and Pearson's correlation efficient was R = −0.69. Both parameters fitted the quantification of the progression of fibrosis from F2 to F4.

LecT-Hepa, Combined with Two Glyco-Parameters, Was Evaluated in the Diagnosis of Significant Fibrosis, Severe Fibrosis, and Cirrhosis. LecT-Hepa was calculated using two glyco-parameters (AOL/DSA and MAL/DSA). The boxplots of LecT-Hepa in relation to the fibrosis staging are shown in Fig. 2. The LecT-Hepa values gradually increased with the progression of fibrosis. Pearson's correlation coefficient between LecT-Hepa and liver fibrosis was very high (R = 0.72), and was superior to those for AOL/DSA (R = 0.61) and MAL/DSA (R = −0.69). We next examined AUC to characterize the diagnostic accuracy of LecT-Hepa 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 (95% CI), sensitivity, specificity, PPV, NPV, LR (+), and LR (−) of the test were 0.802 (0.738-0.865), 59.6%, 89.9%, 85.7%, 66.7%, 5.89, and 0.45,

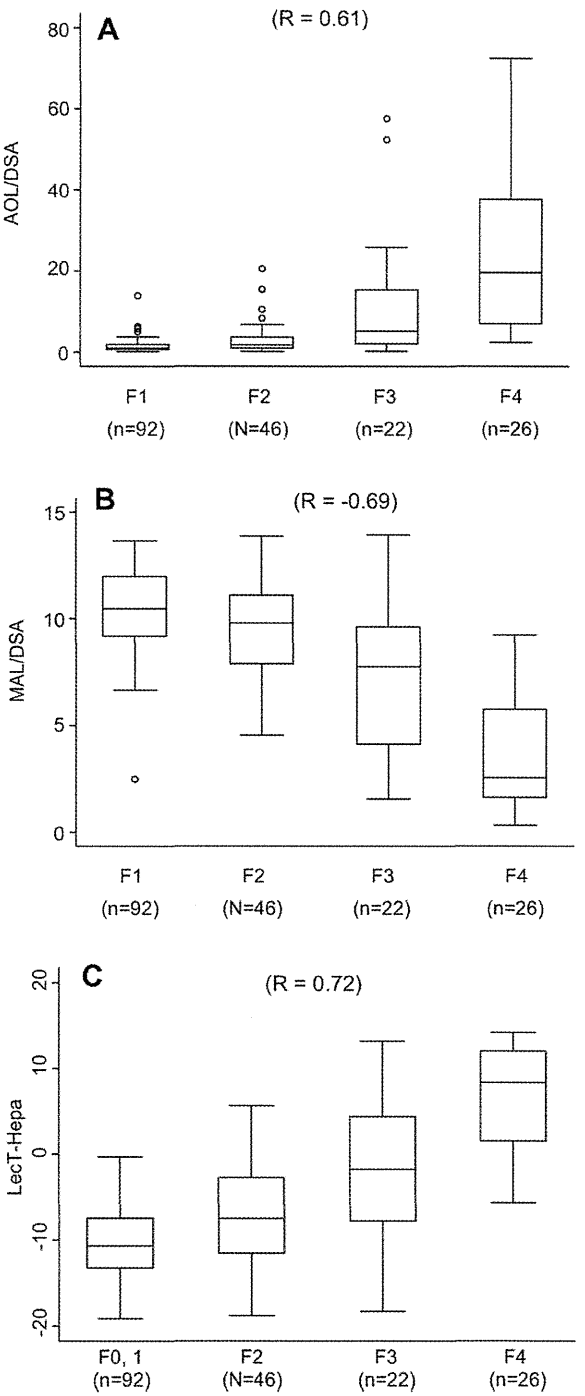
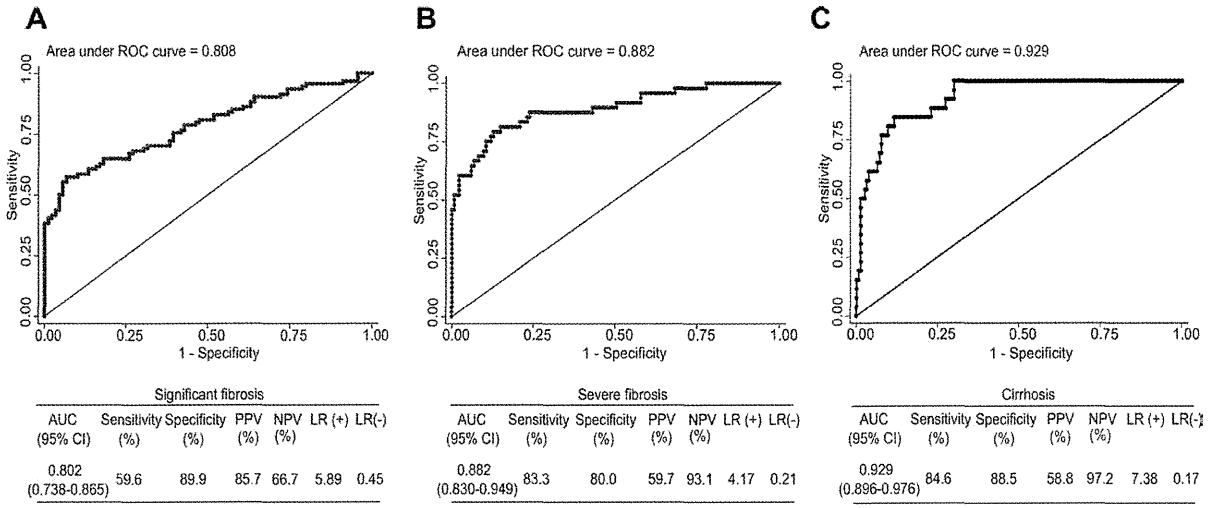


Fig. 1. Boxplot of (A) AOL/DSA, (B) MAL/DSA, and (C) LecT-Hepa in relation to the fibrosis score. The box represents the interquartile range. The whiskers indicate the highest and lowest values, and the dots represent outliers. The line across the box indicates the median value. Correlation of AOL/DSA, MAL/DSA, and LecT-Hepa was measured by HISCL with the progression of liver fibrosis. R: Pearson's correlation coefficient.



Significant fibrosis						
AUC (95% CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	LR (+)	LR(-)
0.802 (0.738-0.865)	59.6	89.9	85.7	66.7	5.89	0.45

Severe fibrosis						
AUC (95% CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	LR (+)	LR(-)
0.882 (0.830-0.949)	83.3	80.0	59.7	93.1	4.17	0.21

Cirrhosis						
AUC (95% CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	LR (+)	LR(-)
0.929 (0.896-0.976)	84.6	88.5	58.8	97.2	7.38	0.17

Fig. 2. ROC curves of LecT-Hepa to distinguish between significant fibrosis and no significant fibrosis in patients with chronic hepatitis C (A); severe fibrosis and no severe fibrosis (B); cirrhosis and no cirrhosis (C). AUC: area under the receiver operating characteristic curve; PPV: positive predictive values; NPV: negative predictive values; LR (+): positive likelihood ratio; LR (−): negative likelihood ratio.

respectively (Fig. 3A). For the prediction of severe fibrosis, AUC (95% CI), sensitivity, specificity, PPV, NPV, LR (+), and LR (−) were 0.882, 83.3%, 80.0%, 59.7%, 93.1%, 4.17, and 0.21, respectively (Fig. 3B). For the prediction of cirrhosis, AUC (95% CI), sensitivity, specificity, PPV, NPV, LR (+), and LR (−) were 0.929 (0.896-0.976), 84.6%, 88.5%, 58.8%, 97.2%, 7.38, and 0.17, respectively (Fig. 3C).

Comparison of AUC, Sensitivity, Specificity, PPV, and NPV for Predicting the Diagnosis of Significant Fibrosis, Severe Fibrosis, and Cirrhosis. ROC curves of LecT-Hepa, HA, TIMP1, platelets, APRI, Forns index, Fib-4 index, and Zeng's score for predicting significant fibrosis, severe fibrosis, and cirrhosis were plotted, as shown in Fig. 3A-C. The AUC of LecT-Hepa for predicting significant fibrosis (0.802) was superior to HA (0.756), TIMP1 (0.697), platelets (0.729), APRI (0.777), Fib-4 index (0.747), Forns index (0.783), and Zeng's score (0.791). For predicting severe fibrosis, AUC of LecT-Hepa (0.882) was superior to HA (0.839), TIMP1 (0.753), platelet count (0.821), APRI (0.840), Fib-4 index (0.811), Forns index (0.861), and Zeng's score (0.863). For predicting cirrhosis, AUC of LecT-Hepa (0.929) was superior to HA (0.866), TIMP1 (0.783), platelets (0.851), APRI (0.787), Fib-4 index (0.856), Forns index (0.887), and Zeng's score (0.853). Sensitivity, specificity, PPV, and NPV by eight noninvasive tests and markers are shown in Table 4. In general, indicators of LecT-Hepa were superior to other noninvasive tests and markers. Specificity and PPV used to distinguish significant fibrosis in LecT-Hepa were superior to those in other tests and markers, although sensitivity and NPV by LecT-Hepa (59.6% and 66.7%, respectively) to distinguish significant fibrosis were inferior to those in other tests and markers. When distinguishing severe fibrosis, the categories of sensitivity (83.3%), specificity (80.0%), PPV (59.7%), and NPV (93.1%) for LecT-Hepa were superior to those in other tests and markers, except for specificity (82.2%) and PPV (61.0%) in HA. When distinguishing cirrhosis, the categories of sensitivity (84.6%), specificity (88.5%), PPV (58.8%), and NPV (97.2%) in LecT-Hepa were superior to those in other tests and markers, except for sensitivity by HA (88.5%), Forns index (84.6%), and Zeng's score (92.3%) and NPV by Zeng's score (98.3%).

Discussion

Our results showed that the LecT-Hepa test, calculated by combining two glyco-parameters (AOL/DSA and MAL/DSA), had higher sensitivity and specificity for diagnosing severe fibrosis and cirrhosis compared to other noninvasive tests and markers for these conditions. The new glyco-marker we have developed is based on the glyco-alteration on the AGP, which is mainly synthesized in the liver. AGP has been considered one of the best candidates for glyco-markers in liver fibrosis or HCC. This is because it is a well-characterized glycoprotein with five highly branched, complex-type *N*-glycans, whose alteration (e.g., desialylation, increased branching, and increased fucosylation) occurs during the progression of liver fibrosis and carcinogenesis.[24] It has already been reported that an

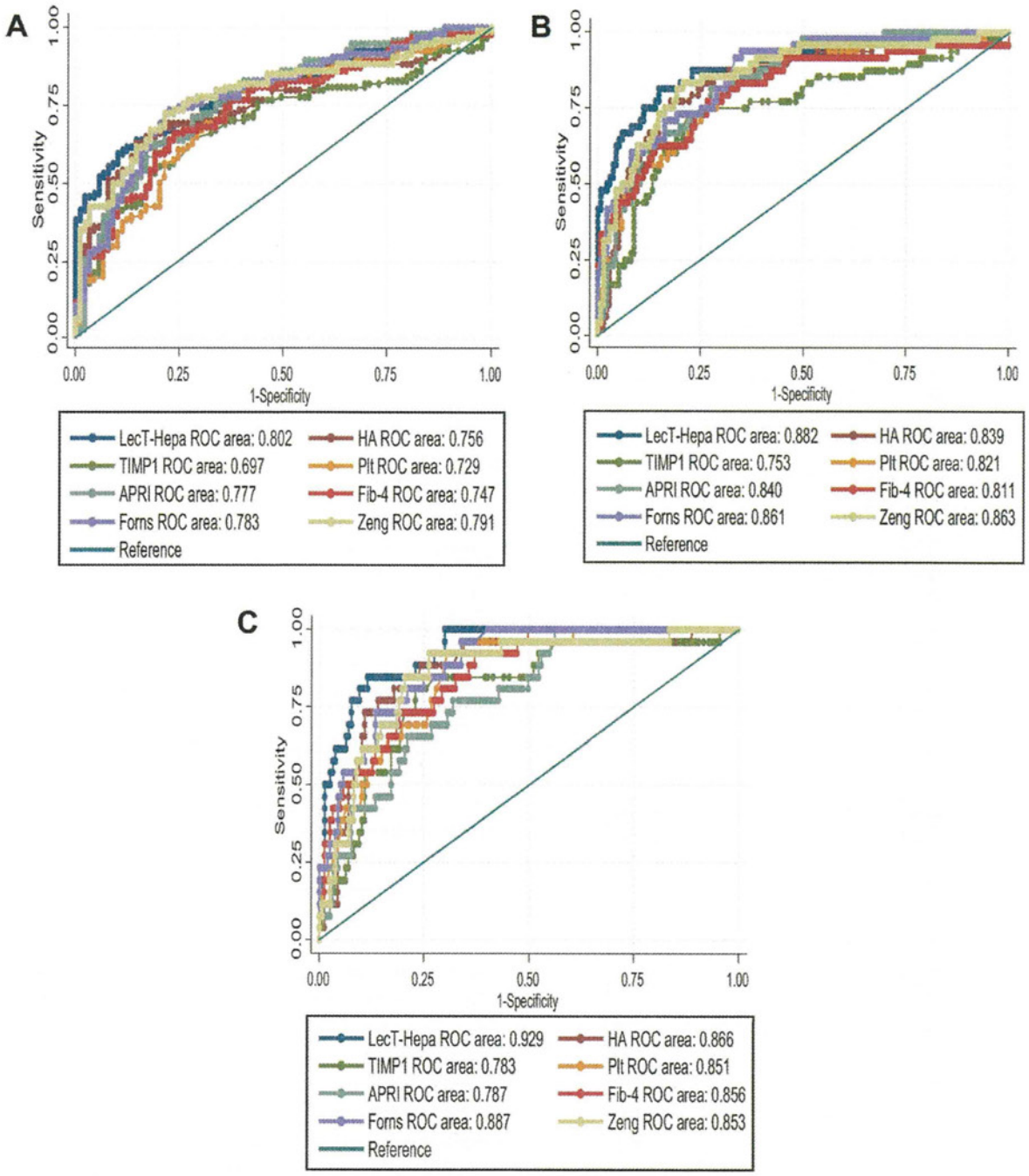


Fig. 3. Comparison of ROC curves in the performance of LecT-Hepa, HA, TIMP1, Plt, APRI, Fib-4 Index, Forns index, Zeng's score for the diagnosis of significant fibrosis (A), severe fibrosis (B), and cirrhosis (C). ROC: receiver operating characteristic curve; TIMP1: tissue inhibitors of metalloproteinases 1; Plt: platelet count; HA: hyaluronic acid.

increased degree of fucosylation was detected in cirrhosis patients using a fucose-binding lectin (AAL)-antibody sandwich ELISA and an automated analyzer.[24] The detection of asialo-AGP using lactosamine-recognition lectin RCA120 has also been reported as an alternative method for finding cirrhosis.[25] Meanwhile, we detected many other aspects of glyco-alteration of AGP using a multiplex sandwich immunoassay with a 43-lectin microarray,[26] resulting in the selection of three lectins—MAL, AOL, and DSA—to serve, collectively, as a fibrosis indicator and a signal normalizer.[14] Since two glyco-parameters (AOL/DSA and MAL/

Table 4. Diagnostic Performance of Biochemical Markers and Scores by Stage of Fibrosis

	No Significant Fibrosis (F0-1) vs. Significant Fibrosis (F2-4)				No Severe Fibrosis (F0-2) vs. Severe Fibrosis (F3-4)				No Cirrhosis (F0-3) vs. Cirrhosis (F4)			
	AUC (95% CI)	Se (%)	Sp (%)	PPV (%)	NPV (%)	AUC (95% CI)	Se (%)	Sp (%)	PPV (%)	NPV (%)	AUC (95% CI)	Se (%)	Sp (%)	PPV (%)	NPV (%)
LecT-Hepa	0.802 (0.738-0.865)	59.6	89.9	85.7	66.7	0.882 (0.830-0.949)	83.3	80	59.7	93.1	0.929 (0.896-0.976)	84.6	88.5	58.8	97.2
HA	0.756 (0.684-0.827)	68.1	78.7	77.8	69.6	0.839 (0.771-0.908)	77.1	82.2	61	90.3	0.866 (0.790-0.942)	88.5	75.8	37.3	96.8
TIMP1	0.697 (0.619-0.774)	65.9	71.9	70.4	60.7	0.753 (0.665-0.841)	75	76.3	53	88.9	0.783 (0.710-0.887)	80.8	74.5	27.8	94.6
Platelets	0.729 (0.656-0.803)	78.7	61.9	68.5	73.5	0.821 (0.751-0.891)	81.3	70.4	49.4	91.3	0.851 (0.785-0.918)	84.6	70.7	32.3	95.8
APRI	0.777 (0.709-0.844)	71.3	71.9	72.2	68.8	0.840 (0.780-0.900)	81.3	72.6	50.6	91.5	0.787 (0.703-0.871)	76.9	68.2	27.9	93.9
Fib-4	0.747 (0.671-0.818)	65.9	76.4	74.7	68	0.811 (0.733-0.889)	77.1	73.3	50	89.2	0.856 (0.788-0.924)	73.1	80.9	37.5	94.1
Forns	0.783 (0.716-0.852)	73.4	77.5	77.5	73.4	0.861 (0.802-0.920)	81.3	71.1	50	91.4	0.887 (0.831-0.943)	84.6	75.2	36.1	96.7
Zeng	0.791 (0.723-0.858)	82.9	70.7	75	79.7	0.863 (0.799-0.925)	81.3	79.8	59.5	92.8	0.853 (0.783-0.933)	92.3	73.9	36.9	98.3

AUC, area under the ROC curve; CI, confidence interval; Se, sensitivity; Sp, specificity; PPV, positive predictive values; NPV, negative predictive values.

DSA) on AGP are normalized by an internal standard lectin (DSA), LecT-Hepa is not influenced by the amount of AGP. We confirmed that the use of this lectin set was statistically superior to the previously selected lectins (AAL and RCA120).

This triplex-sandwich immunoassay employing DSA/MAL/AOL lectins and an anti-AGP antibody from the lectin microarray has already been converted to a fully automated immunoassay analyzer (HISCL-2000i) for clinical use.[15] Pretreatment requires 3 hours, and quantifying the two glyco-parameters for the LecT-Hepa to use this automated analyzer takes 17 minutes. Currently, we can obtain data from LecT-Hepa to predict liver fibrosis on the same day of blood sample collection. This simple and reliable glyco-marker may be suitable for clinical use, and may substitute for liver biopsy in some cases.

We are confident that our study samples are representative of most patients. The AUC scores for distinguishing significant fibrosis, severe fibrosis, and cirrhosis by APRI, HA, Fib-4 index, Forns index, and Zeng's score were not significantly different from those in previous studies.[11,27,28] Every serum sample in this study was obtained from a patient immediately before or no more than 2 months after liver biopsy. As many serum samples as possible were collected from each liver center to eliminate a selection bias in any center. Since we could not perform liver biopsy on the patients who had a tendency to develop hemorrhages, fewer samples of severe fibrosis and cirrhosis were collected than those of milder fibrosis. In fact, the population of fibrosis staging in this study was similar to that of a previous, large prospective study evaluating noninvasive fibrosis markers.[29] In addition, we did not include patients with obvious decompensated cirrhosis. This is because inclusion of patients with severe liver disease would have artificially improved the predictive values of the logistic function. On the other hand, we included many patients with mild histological features (48.6% with F0-1). Sampling variation poses potential difficulties, especially in the early stages of disease, when fibrosis might be unevenly distributed.

There are several advantages in using reliable noninvasive markers for assessing liver fibrosis. First, they can be used to accurately determine the appropriate time for initiating IFN treatment in CHC patients. These markers can also help monitor and assess the therapeutic efficacy of IFN treatment in improving liver function in cases of liver fibrosis and cirrhosis. Finally, these markers will be essential in the development of new, antifibrotic treatments. Recently, many directed or targeted therapies against liver fibrosis,

such as anti-transforming growth factor beta and anti-tumor necrosis factor alpha compounds have been developed.[30,31] To evaluate these new drugs, reliable and simple noninvasive fibrosis markers are needed. LecT-Hepa appears to be one of the most prominent candidates to serve as a marker for developing antifibrotic drugs.

In conclusion, both glyco-parameters (AOL/DSA and MAL/DSA) using lectins in a bedside, clinical chemical analyzer succeeded in the quantification of the progression of liver fibrosis. Using LecT-Hepa, the combination score of both AOL/DSA and MAL/DSA is a reliable method for determining fibrosis staging and can be a good substitute for liver biopsy.

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Lectin microarrays: concept, principle and applications†

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The lectin microarray is a novel platform for glycan analysis, having emerged only in recent years. Unlike other conventional methods, *e.g.*, liquid chromatography and mass spectrometry, it enables rapid and high-sensitivity profiling of complex glycan features without the need for liberation of glycans. Target samples include an extensive range of glycoconjugates involved in cells, tissues, body fluids, as well as synthetic glycans and their mimics. Various procedures for rapid differential glycan profiling have been developed for glycan-related biomarkers. Such glycoproteomics targeting allows precise diagnosis of chronic diseases potentially related to cancer. Application of this method to evaluation of various types of stem cells resulted in the discovery of a new pluripotent cell-specific glycan marker. To explore this technology a more fundamental and extensive understanding of lectins is necessary in relation to the structural uniqueness of glycans. In this chapter, the essence of the lectin microarray is described with some focus on an evanescent-field-activated fluorescence detection principle as a system to achieve *in situ* (*i.e.*, washing free) aqueous-phase observation under equilibrium conditions. The developed lectin microarray system allows even researchers with poor experience in glycan profiling to perform extensive high-throughput analysis targeting various forms of glycans and even cells.

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† Part of the carbohydrate chemistry themed issue.

1. Unique properties of glycans

One of the most prominent features of glycans, which distinguishes them from other major biopolymers such as nucleic acids and proteins, is "heterogeneity". The difference is largely attributed to their basic fabrication. The number of elementary sugars

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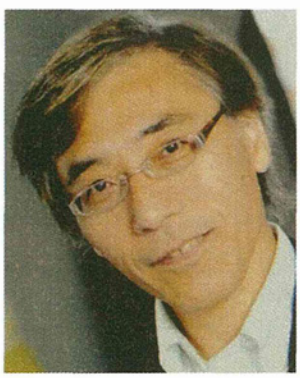

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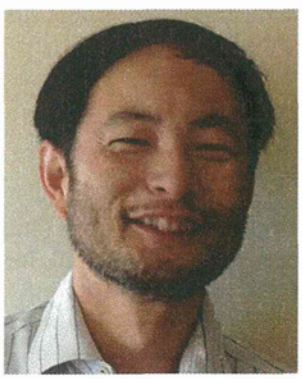
(monosaccharides) found in natural glycans is rather moderate. There are many monosaccharide hexoses and pentoses essentially absent in nature. Chemical approaches, such as the cyanohydrine method,[1] enable synthesis of the full number of theoretically-possible isomers of simple D-aldohexoses. However, only some 10 monosaccharides represent naturally-occurring elementary monosaccharides in mammals: D-glucose (D-Glc), D-mannose (D-Man), D-galactose (D-Gal), *N*-acetylglucosamine (D-GlcNAc), *N*-acetylgalactosamine (D-GalNAc), D-glucuronic acid (D-GlcA), L-fucose (L-Fuc), *N*-acetylneuraminic acid (Neu5Ac), D-xylose (D-Xyl). In non-human vertebrates, *N*-glycolylneuraminic acid (Neu5Gc) is also utilized, while plant and bacterial glycans contain L-arabinose (L-Ara) and L-rhamnose (L-Rha) as common saccharides, respectively. As regards the question of how these elementary saccharides were selected, a hypothesis has been presented by one of the authors, in which Fru, Glc and Man are regarded as the first triplet hexoses generated as a result of chemical evolution, while other monosaccharides including Gal, deoxyhexoses, sialic acids and pentoses including ribose are late-arriving, "bricolage" products derived from Glc and Man.[2]

Glycans are often branched, because glycosidic bonds are formed between the anomeric OH group of a non-reducing terminal monosaccharide and any of the multiple (*e.g.*, 4 in the case of aldohexoses) non-anomeric 2-, 3-, 4-, 6-OH groups of the reducing terminal monosaccharide. The situation is complicated further by the possible formation of the α- or β-anomeric form of the glycosidic linkage. Thereby, the theoretical number of branched and non-branched hexasaccharides becomes as large as 1.05×10^{12}.[3] Thus many glycosidic bonds are theoretically possible but do not necessarily occur naturally for at least two reasons: (i) the occurrence of steric hindrance between the two relevant monosaccharides in terms of dihedral angles ϕ, ψ and ω[4] and (ii) the absence of relevant enzymes (*i.e.*, glycosyltransferases) to catalyze glycoside bond formation, which require high substrate specificity.[5]

Another element that further increases the structural complexity of glycans is the variety of glycoconjugates, *i.e.*, glycoproteins, glycolipids and proteoglycans, though free glycans exist in some cases, such as in milk.[6] In the case of glycoproteins, further complexity arises in terms of both structural types (*i.e.*, *O*-glycans, *N*-glycans) and multiple attachment sites, each of which may be glycosylated or non-glycosylated. All these elements increase the substantial diversity of glycoconjugates still more. For these reasons, all of the conventional procedures for the analysis of glycoprotein glycans, represented by high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), mass spectrometry (MS), or their combinations, require prior liberation of the attached glycans from their core proteins.[7] In addition, due to lack of appropriate UV absorption in the liberated glycans, they are usually fluorescently labeled for the purpose of both detection and the improvement of separation in HPLC. Fluorescence labeling also improves ionization efficiency in MS. For these reasons, the total process required for glycan analysis is necessarily complicated, laborious, and time-consuming.

2. Alternative approach to glycan analysis using lectins

How then can we approach glycan analysis in the absence of automated systems for sequencing and synthesis? Conventional methods (*e.g.*, LC, CE, MS) require both prior liberation and subsequent labeling. On the other hand, a series of carbohydrate-binding proteins, or lectins, has long been used in a more direct manner for "glycan profiling", *e.g.*, lectin-probed flow cytometry, histochemical staining, Western blot analysis, and glycoprotein fractionation using lectin-affinity chromatography.[8,9] Other methods based on specific detection principles are also available, *e.g.*, surface plasmon resonance (SPR) and quartz crystal microbalance (QCM). Especially, these methods provide a useful



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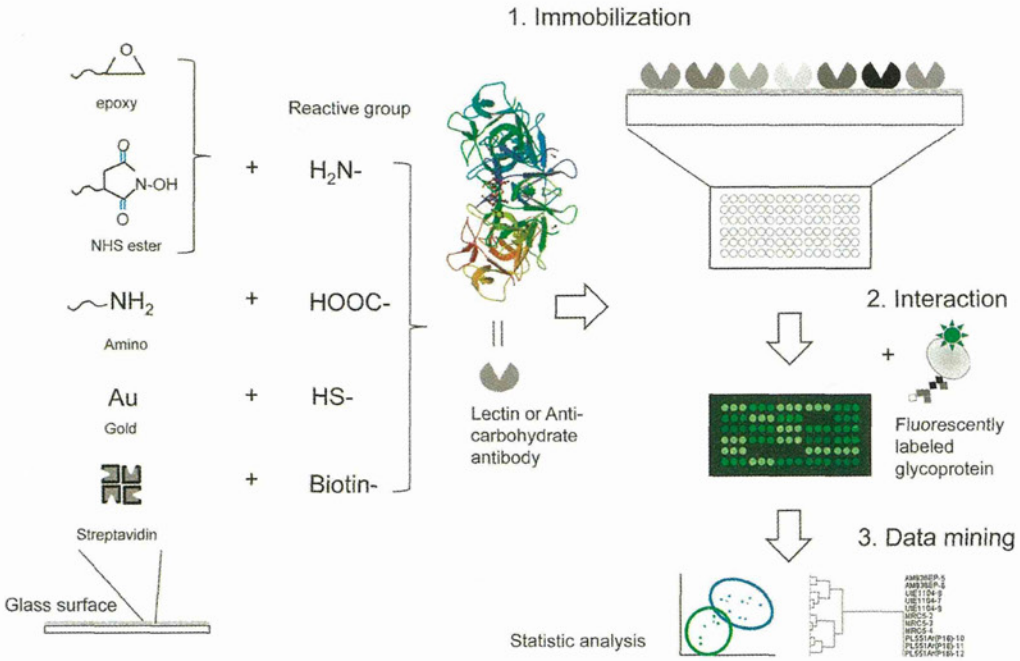

Fig. 1 A general scheme to make lectin microarray substrates and subsequent procedures for glycan profiling. A series of lectins (or anti-carbohydrate antibodies) is immobilized on a glass substrate, the surface of which is activated by various chemical (*e.g.* epoxy, *N*-hydroxysuccinimide (NHS), amino, gold) or biochemical (*e.g.* streptavidin) derivatization procedures. To the derived lectin microarray substrate, fluorescently labelled glycoproteins (or cell/tissue extracts containing them) are added to promote the binding reaction sufficiently. Under equilibrium conditions, *i.e.* no washing, the bound glycoproteins are quantified as signal intensities in each of the lectin spots. The quantified data thus obtained are used for data analyses including statistics.

approach to quantitative protein–ligand interaction analysis in a label-free manner (for recent reviews, see ref. 10 and 11, respectively). Although these conventional techniques are useful, unfortunately they lack "throughput" comparable to current omic studies.

In 2005, a novel technique called the lectin microarray was developed almost simultaneously by different groups.[12–15] In general, a series of lectins and carbohydrate-binding antibodies with various carbohydrate-binding specificity are immobilized on a glass slide through appropriate surface chemistry, *i.e.*, between either epoxy-, NHS (*N*-hydroxysuccinimide)-, amino-, or biotin-modified glass surface and their counter functional groups of lectin proteins (Fig. 1). After the immobilization procedure, surface areas containing residual activated groups are treated with appropriate blockers, such as an amine (*e.g.*, Tris base) or glycan-free serum albumin. Binding of target glycoproteins can be detected either directly through their prior labeling with fluorescent reagents (*e.g.*, Cy3-NHS), or indirectly by overlaying a fluorescently labeled relevant antibody raised against the target glycoprotein (or *via* biotinylated antibody and fluorescently labeled streptavidin). For the latter, the target glycoprotein must be selected in advance with a suitable, available antibody.[16]

Since the first publication, the lectin microarray technology has attracted increasing attention of not only glycoscientists but also researchers in other fields. This is particularly because the method enables direct analysis of even crude samples containing glycoproteins, without liberation of glycans, unlike conventional methods.[7] Nevertheless, one should keep in mind that the technique is not quantitative and does not allow complete determination of glycan structures as MS can, though matching of identical glycan profiles can fall into the range of this technology. Rather, the method is more efficiently applied for comparative purposes, such as differential profiling. Concept, strategy, and technical advancement of the lectin microarray have been described by various investigators[17–19] including the authors of this review.[7,20] It should also be mentioned that some other related microarray technologies in glycobiology are available: these include a glycan microarray (a topic of this themed issue) and a lectin-overlay antibody microarray (Fig. 2).

3. Paradox of lectin-based glycomics

One might think that lectins are still inferior to anti-carbohydrate antibodies in terms of specificity and affinity. However, this idea should be assessed from various aspects as previously discussed by Gemeiner *et al.*[21]: *first,* the precise affinity of an anti-carbohydrate antibody is not determined systematically, while those to other antigens have high affinity ranging from 10^{-9} to 10^{-7} M in terms of the dissociation constant (K_d) (Table 1). *Second*, anti-carbohydrate antibodies, if anything, show relatively low affinity and even low specificity to target glycans. This is basically because most of them are IgM but not IgG, attributable to the fact that most carbohydrate structures are common to host animals (*e.g.*, mice and rabbits), and thus, not antigenic.

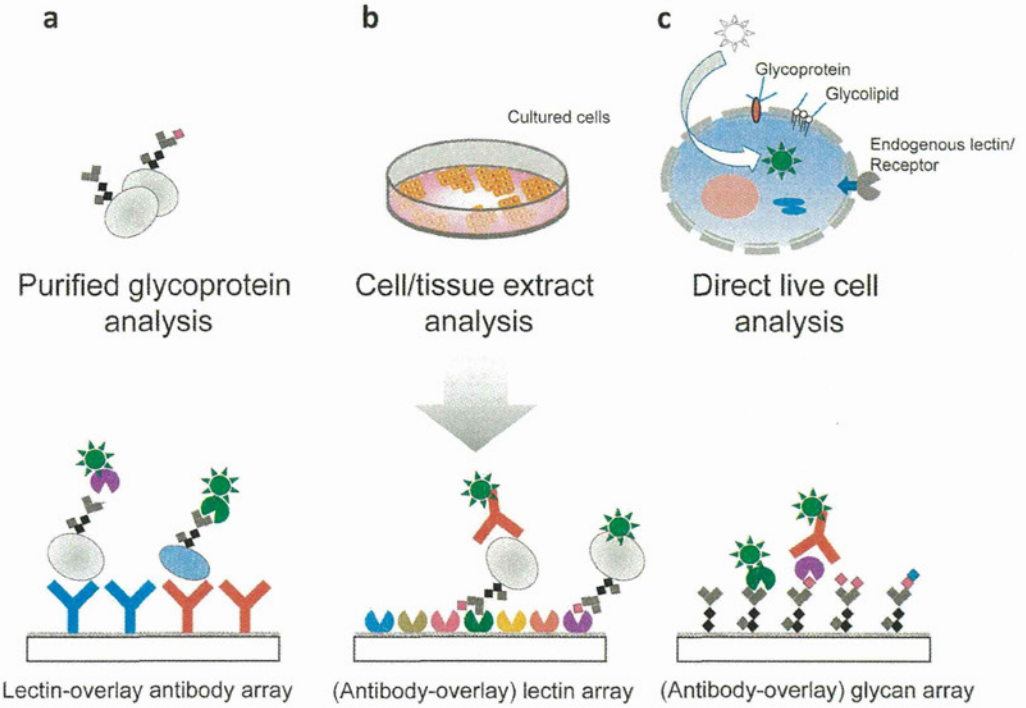

Fig. 2 A variety of microarray formats generally used for glycan profiling. (a) A lectin-overlay antibody microarray, in which a series of antibodies is immobilized on the glass slide and target glycoproteins are captured. Glycan structures of the captured glycoproteins are analyzed by multiple previously-labelled lectin probes. (b) The lectin microarray is described in detail in the text: a series of lectins is immobilized and target glycoproteins are detected either directly (prior labelling) or indirectly with an overlaying labelled antibody for a target glycoprotein. (c) The glycan microarray generally comprises a large number (>100) of synthetic and naturally obtained glycans and their derivatives, to which binding of various types of glycan-binding proteins (*i.e.*, lectins and anti-carbohydrate antibodies) is analyzed either directly (prior labelling) or indirectly (antibody-overlay procedure).

Table 1 Ranges of affinity of various bio-molecular interactions in terms of the dissociation constant (K_d)

Group of bio-molecules	Range of affinity (K_d)
Lectin–oligosaccharide (Lectin–glycoprotein with multiple glycosylation sites)	10^{-7}–10^{-3} M ($<10^{-7}$ M)
Complex salt	10^{-8}–10^{-2} M
Protein A–IgG	10^{-6} M
Antigen–antibody (monoclonal)	10^{-9} –10^{-7} M
Hormone–receptor	10^{-12}–10^{-5} M
Avidin–biotin	10^{-15} M
Covalent bond	10^{-60} M

In fact, there has been no report of IgG being successfully raised against evolutionarily conserved *N*-linked glycans. On the other hand, some xenoantigenic glycan epitopes are known, which include the α-Gal epitope[22] and glycolyl-type sialic acid (Neu5Gc[23]). It is worth reevaluating anti-carbohydrate antibodies through detailed analysis of their specificity and affinity in comparison with other antibodies.

Third, and most importantly, such rigorously specific anti-carbohydrate antibodies, if any, could never work for the purpose of comprehensive glycan profiling. Although it is difficult to estimate the actual size of the glycome, it would certainly exceed the order of 10^3–10^4 including all levels of structural diversity. If this is the case, how do life systems discriminate subtle differences in such a large complexity expressed on the cell surface, extracellular spaces and glycoproteins secreted in body fluids? Therefore, if only for economic reasons, it is impossible to prepare a set (>10 000) of antibodies to identify each of the probable glycans. Instead, a repertoire of glycan binding proteins (both lectins and available antibodies) will work for this purpose, because they have relatively low affinity, and thus, broad specificity. The latter fact means that wider coverage is attainable by each lectin, and the combination of multiple lectins will cover substantially almost all glycome sizes. The number of lectins required for precise profiling will depend on the glycome size. In fact, numerous lectins have been identified in almost all organisms, including viruses. It is rather surprising that many lectins function as useful tools for the analysis of human cells and the glycoproteins they produce, despite the fact that most of the probe lectins are exogenous, *i.e.*, derived from plants and microorganisms.

4. Representative lectins used for glycan profiling

For many reasons (historical, commercial availability, stability, economy, a variety of specificities, *etc.*), plant lectins are widely used. They include the families of legume lectins[24] and ricin B chain-like (R-type) lectins,[25] both of which show a wide range of specificities. The jacalin-related lectin family consists of Man-specific type and Gal-specific type lectins even in the same