Table 2 Factors associated with HCC development

		Univariate		Multivariate	
		HR (95% CI)	*P*	HR (95% CI)	*P*
Age (every 10 years)		2.0 (0.8–5.0)	0.1		
Sex (male/female)	Male	1			
	Female	0.4 (0.1–1.2)	0.1		
AST (IU/L)	<40	1			
	≥40	2.5 (0.6–11)	0.2		
ALT (IU/L)	<40	1			
	≥40	1.3 (0.4–3.7)	0.6		
Bilirubin (mg/dL)		2.8 (0.3–24)	0.3		
Platelet counts (×10^9/L)	≥150	1			
	<150	2.4 (0.6–8.4)	0.2		
Fibrosis stage	F1/2	1		1	
	F3/4	3.9 (1.3–11)	0.01	1.8 (0.5–6.3)	0.3
AFP (ng/mL)	<10	1		1	
	≥10	5.8 (1.8–18)	0.003	4.7 (1.1–19)	0.03
WFA$^+$-M2BP (COI)	<4.2	1		1	
	≥4.2	8.2 (2.6–26)	<0.001	4.1 (1.1–15)	0.04
ΔWFA$^+$-M2BP/year	<0.3	1		1	
	≥0.3	3.1 (1.1–9.3)	0.04	5.5 (1.5–19)	0.008

AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CI, confidence interval; COI, cut-off index; HCC, hepatocellular carcinoma; HR, hazard ratio.

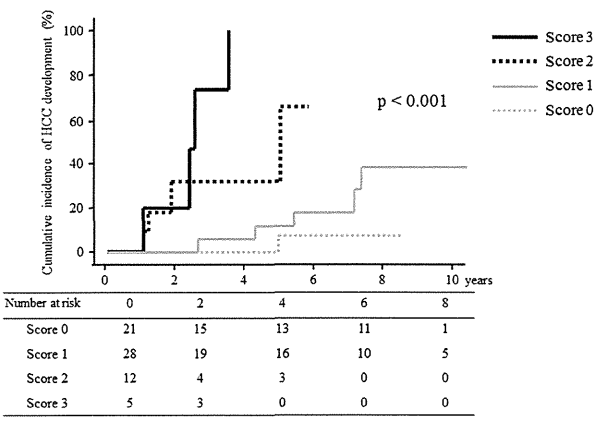

Figure 4 Association between the risk score and cumulative incidence of hepatocellular carcinoma (HCC) development. WFA$^+$-M2BP of 4.2 or more, ΔWFA$^+$-M2BP/year of 0.3 or more and AFP of 10 ng/mL or more each contributed 1 point to the score. WFA$^+$-M2BP of less than 4.2, ΔWFA$^+$-M2BP/year of less than 0.3 and α-fetoprotein (AFP) of less than 10 ng/mL each contributed 0 points to the score. Patients were classified into four groups according to the total score of 0, 1, 2 or 3.

to be a significant predictive factor for HCC development. In a recent study, the significance of WFA$^+$-M2BP for prediction of HCC was demonstrated in a large cohort study, further confirming the clinical impact of WFA+-M2BP.[28]

A new finding of our study was that time-course changes in WFA$^+$-M2BP were associated with HCC development. An advantage of WFA$^+$-M2BP testing over liver biopsy is that its non-invasiveness is suitable for repeated measurement. Liver biopsy is problematic to repeat to assess time-course changes because of its invasiveness.[29] WFA$^+$-M2BP quantification can be used for real-time monitoring of liver disease, based on our finding that time-course changes were associated with HCC development. Furthermore, WFA$^+$-M2BP and time-course changes in WFA$^+$-M2BP were independent predictors of HCC development, and patients at high risk of HCC development could be identified using a combination of these factors. Therefore, single-point WFA$^+$-M2BP assessment plus time-course changes in WFA$^+$-M2BP are more useful to predict HCC development than a single-point liver biopsy.

WFA$^+$-M2BP has some advantages over other serum fibrosis markers and elastography. Although APRI and FIB-4 serum fibrosis markers have demonstrated utility in predicting HCC development,[19–21] they are calculated using AST, ALT, platelet count and age. Hence, APRI and FIB-4 may not be appropriate in cases of advanced age, fatty liver or interferon therapy.[30] Furthermore, diagnostic accuracy of APRI and FIB-4 for HCC development was inferior to WFA$^+$-M2BP in this study.

Liver elastography using ultrasonography has utility in predicting HCC development as well,[16] but these

modalities are not widely available, particularly in resource-constrained settings. Furthermore, measurements may be impossible in patients with severe obesity or ascites.[31] Reproducibility of transient elastography may be impaired in patients with steatosis, increased body mass index or less severe liver fibrosis.[32] In contrast, WFA$^+$-M2BP quantification requires a small blood sample and WFA$^+$-M2BP can be accurately measured without interference from the previously mentioned factors. WFA$^+$-M2BP quantification is entirely automated using the HISCL-2000i system and results can be acquired within 17 min. Because of these advantages, WFA$^+$-M2BP is more useful to predict liver fibrosis and HCC development than other serum fibrosis markers or elastography.

Our study was limited by the small number of patients and case–control pilot design. Patient characteristics between two groups were matched, but age, sex and fibrosis stage were biased nevertheless. A larger prospective study is needed to evaluate the utility of WFA$^+$-M2BP and time-course changes in WFA$^+$-M2BP as predictive factors of HCC development.

In conclusion, WFA$^+$-M2BP and time-course changes in WFA$^+$-M2BP were found to be independent predictive factors of HCC development, and patients at high risk of HCC development could be identified by combining these factors into a scoring system. Because WFA$^+$-M2BP quantification can be easily repeated, real-time monitoring of WFA$^+$-M2BP could be a novel predictor of HCC development.

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CONFLICT OF INTEREST

THE AUTHORS WHO have taken part in this study declare that they have no conflicts of interest to disclose.

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Development and Applications of the Lectin Microarray

Jun Hirabayashi, Atsushi Kuno, and Hiroaki Tateno

Abstract The lectin microarray is an emerging technology for glycomics. It has already found maximum use in diverse fields of glycobiology by providing simple procedures for differential glycan profiling in a rapid and high-throughput manner. Since its first appearance in the literature in 2005, many application methods have been developed essentially on the same platform, comprising a series of glycan-binding proteins immobilized on an appropriate substrate such as a glass slide. Because the lectin microarray strategy does not require prior liberation of glycans from the core protein in glycoprotein analysis, it should encourage researchers not familiar with glycotechnology to use glycan analysis in future work. This feasibility should provide a broader range of experimental scientists with good opportunities to investigate novel aspects of glycoscience. Applications of the technology include not only basic sciences but also the growing fields of bio-industry. This chapter describes first the essence of glycan profiling and the basic fabrication of the lectin microarray for this purpose. In the latter part the focus is on diverse applications to both structural and functional glycomics, with emphasis on the wide applicability now available with this new technology. Finally, the importance of developing advanced lectin engineering is discussed.

Keywords Bio-affinity • Biomarker • Functional glycomics • Glycan profiling • Lectin microarray

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Contents

1 Why Glycomics with Lectins?

1.1 Inherent Properties of Glycans

Glycosylation endows a protein with various enhanced properties which the naked protein itself does not possess. Examples of these additional properties in protein glycosylation include polypeptide folding, stability, solubility, destination, functional regulation, and efficiency. In other words, glycosylation makes the role of a protein multidimensional [1, 2]. However, investigating protein glycosylation is not simple experimentally. Glycan structures are extremely diverse, for instance, in comparison with protein phosphorylation, another common post-translational modification. In general, glycans found in natural glycoproteins of higher animals consist of aldohexoses (e.g., Glc, Man, and Gal), their *N*-acetyl derivatives (e.g., GlcNAc and GalNAc), and, in many cases, deoxyhexose (e.g., L-fucose) and sialic acids represented by *N*-acetylneuraminic acid (NeuAc). An aldopentose, xylose, is also a common constituent of proteoglycan, and is involved in a linker region between core protein and glycosaminoglycan chains, such as heparin, heparan sulfate, dermatan sulfate, and chondroitin sulfate. However, the complexity of glycans is not strongly attributed to diversity in such component saccharides but more significantly to that in linkage isomers, because each hexose monosaccharide (e.g., Glc) possesses four potential donor hydroxyl groups (2-, 3-, 4-, and 6-OH) and one acceptor 1-hemiacetal (O–C–OH). The presence of anomerism (α/β) further doubles the number of linkage isomers. Indeed, there are eight possible isomers to

link two identical aldohexoses (e.g., Glc): "Glcα1-2/3/4/6Glc" and "Glcβ1-2/3/4/6Glc." The presence of multiple linkage isomers also allows branching in glycans, which is completely absent in nucleic acids and proteins. In theory, six monosaccharides can make as many as 1.05×10^{12} molecules of structural complexity, according to Laine [3]. Apparently, this figure far exceeds those calculated for hexanucleotides (i.e., $4^6 = 4{,}096$) and hexapeptides (i.e., $20^6 = 64{,}000{,}000$). Nobody knows the actual size of the glycome. However, it is considered that complete separation technology for glycans is not available. It also appears unrealistic currently to develop an automated method for glycan sequencing or synthesis [4, 5].

Because of the indirect association between genome and glycome, it is also difficult to predict glycan structures merely from the gene expression profiles: other information is necessary about the amounts of sugar nucleotide donors and location of a series of glycosyltransferases in the Golgi apparatus, some of which may compete with one another. However, the most important fact and the largest obstacle, which is never overcome by the classic concept workable for nucleic acids and proteins, is "heterogeneity" of glycans, partly because glycan biosynthesis is achieved only as a result of consecutive steps of glycosyltransferase and glycosidase reactions. These biosynthetic reactions can be incomplete or compete with one another. The extent of heterogeneity is further increased at the level of glycoconjugates (e.g., glycoproteins), because glycan structures and occupancies are not always the same between individual glycosylation sites. Of course, glycan structures should change if cell types and states that produce glycoproteins are different, even if the core protein structure is the same. This basic principle of glycoproteomics has been successfully applied to glycoprotein biomarker development for better diagnostic molecules [6, 7].

1.2 Lectin Microarray, an Advanced Technology for Glycan Profiling

Recent advances in separation and analytical technologies are noteworthy. They are represented by high-performance liquid chromatography (HPLC) and mass spectrometry (MS) as well as their combination with other separation principles (e.g., capillary electrophoresis, affinity chromatography). As described, however, it is still difficult for these methods to distinguish completely diverse structural isomers, and all of these conventional methods assume prior liberation of glycans and in most cases their separation and labeling. For the latter purpose, appropriate fluorescent tags (e.g., 2-aminopyridine and 2-aminobenzamide) are used because of increased sensitivity and resolution in HPLC. However, these processes require substantial labor and significant sacrifice of "throughput." Although a commonly-used method for glycan liberation is an enzymatic cleavage of glycans from the core proteins, the procedure has substantial limitation to a class of asparagine-linked

N-glycans. On the other hand, no universal glycosidase has been available for serine/threonine-linked *O*-glycans. As a more fundamental issue, some important biological aspects, e.g., density, depth, and orientation, as well as combination to other biochemical components (e.g., core protein, with which glycan functions are closely associated), would be lost if glycans were to be liberated from the core protein. Mucins are a representative case of such glycoconjugates.

Most conventional methods for glycomics are based on physicochemical principles. However, as emphasized previously, they necessitate separation of "liberated" glycans. This procedure depends largely on the type of glycosylation (i.e., *N*-glycomics, *O*-glycomics, glycosaminoglycomics, glycolipidomics, etc.). Obviously, this is a disadvantage for glycomics if the intention is the comprehensive analysis of all types of glycans/glycoconjugates in a comparative manner. In contrast to these conventional technologies, an alternative is to use biochemical "probes" to glycans, which have a substantial affinity to glycans/glycoconjugates, such as lectins and carbohydrate-binding antibodies. Indeed, various techniques using these glycan probes have already been shown to work in biochemical and cytochemical studies, e.g., lectin-probed western blot analysis, histochemistry, and flow cytometry. While these methods are widely applicable to crude biological samples (e.g., cells and sera), they have a great disadvantage in throughput, sensitivity, and speed, all of which are critical requisites for glycomics. An advanced method, lectin microarray, was developed in 2005 to overcome these drawbacks [8–13]. Lectin microarray has a platform similar to that of antibody microarray, consisting of multiple probes (lectins and carbohydrate-binding antibodies) on an appropriate array substrate (typically a glass slide), which enables simultaneous interaction analyses in an ultrasensitive and high-throughput manner. However, a special attribute of the lectin microarray is differential glycan profiling rather than identification of particular target molecules. It is essentially difficult for the lectin microarray to identify individual glycan structures [4, 14, 15]. Differential glycan profiling can also target a mixture of glycoproteins and clinical samples [16, 17]. Readers can find the essential aspects of the lectin microarray in recent comprehensive reviews (e.g., [18–21]). Therefore, the following are descriptions with a focus on original aspects of the lectin microarray developed in the authors' laboratory.

1.3 Lectins or Carbohydrate-Binding Antibodies?

According to the classic definition, lectins are carbohydrate-binding proteins, with the exception of antibodies and enzymes [22]. However, this criterion should be reconsidered in the light of current understanding of lectins: indeed, siglecs, a group of animal lectins specific for sialic acid, are members of the immunoglobulin superfamily, and an increasing number of enzymes have been found to contain carbohydrate-recognition domains (CRD) other than catalytic domains. On the other hand, it is now evident that even major lectin families, e.g., C-type lectins

and galectins, contain significant numbers of "non-lectin" members. In this context, it seems more reasonable to regard lectins simply as "a group of proteins that have significant ability to bind carbohydrates regardless of the protein families to which they belong." The number of lectins identified is rapidly increasing as glycotechnologies to prove lectin activity have improved [23–25]. Currently, the number of established lectin families exceeds 40, and this number will probably increase significantly in the future. Moreover, it is possible to create artificial lectins not only from natural lectins that have sugar-binding activity but also from those that have no such activity. In other words, all the existing proteins are able to become "novel" lectins if they have potential binding pockets and if they are modified appropriately to make up a binding network to target carbohydrates, e.g., through hydrogen bonds and van der Waals contacts. Another requisite to evolve lectins might be multivalency in various terms; e.g., tandem repeat of CRDs or binding modules, subunit oligomerization, and acquisition of subsite specificity. Siglecs are an example of novel lectins, which have evolved from the immunoglobulin superfamily [26]. The opposite probably applies to galectins-10, 11, 13, and 15, because they have no evident β-galactose-binding activity despite their original definition [27].

As described, both antibodies and lectins are useful tools for glycan profiling. However, one should keep in mind that protein structures are significantly different between species, e.g., human and mouse, whereas many glycan structures are common between these species. For instance, high-mannose structures of *N*-linked glycans are strongly conserved in eukaryotes (e.g., mammals) and, thus, are not antigenic in any animal. On the other hand, representative xenoantigens, the α-Gal epitope and *N*-glycolylneuramic acid (NeuGc) are absent in humans and, thus, are antigenic. However, these xenoantigens are rather exceptional among a large number of common structures. This is a basic reason why only relatively poor anti-carbohydrate antibodies are produced in animals, most of which are attributed to IgM. However, for the sake of differential glycan profiling, rigorously specific antibodies do not work. Rather, probes such as conventional lectins with broader specificity are desirable to ensure the "coverage" of the glycome. Importantly, many of the conventional lectins show significant cross-affinity to structurally-related glycans, but to different extents. On the other hand, in order to detect specific structures (epitopes), carbohydrate-binding lectins or antibodies with rigorously defined specificity should be effective as epitope detectors [28]. The method is also expected to provide an extremely high-throughput means for glycan profiling.

1.4 Preceding Techniques

A previously-developed technique called "serial lectin-affinity chromatography" is available. Although the technique is almost identical to the lectin microarray in its essence, it lacks throughput and speed, because it utilizes open columns and

radio-labeled oligosaccharides [29, 30]. Frontal affinity chromatography (FAC) is a more quantitative method for the analysis of lectin-glycan interactions [31]. The method was recently greatly improved in speed, throughput, and accuracy [32, 33]. Nevertheless, the method is essentially applicable to only purified oligosaccharides. On the other hand, the lectin microarray has a more suitable platform providing simultaneous interaction analyses with a panel of carbohydrate-binding proteins. Moreover, attaining high sensitivity is assured by utilizing an established procedure for labeling proteins with common fluorescent reagents (e.g., Cy3, Cy5). Although special equipment is required for reproducible analysis, especially of monovalent glycans, in many cases lectin-glycoprotein interactions are strong enough to tolerate even repeated washing [8, 9, 11–13]. However, in the case of glycoproteins having only a few glycan chains, and thus relatively weak affinity to a lectin, i.e., in the order of 10^{-3} to 10^{-5} M in dissociation constant (K_d), high-wash procedures after a probing reaction should remove the weak binding. In this case, it is advisable to take advantage of an evanescent-field activated fluorescence detector, which enables in situ observation of immobilized lectin-glycan/glycoprotein interactions while preserving equilibrium [10, 34]. However, substantial affinity enhancement is often observed on an appropriate array platform, particularly when multiple lectin–glycan interactions occur. This phenomenon is generally understood as a glycoside cluster effect, first demonstrated by Y. C. Lee [35], and is closely associated with biological functions of lectins under physiological conditions [36].

2 Basic Fabrication of the Lectin Microarray System

2.1 Equipment

Figure 1 shows a general scheme for glycan profiling using the lectin microarray. Commercial products for this purpose are available [37]: the system consists of the scanner GlycoStation™ Reader 1200 and LecChip™, both of which are produced by GP Biosciences, Co. Ltd. (Yokohama). An advantage is that the system utilizes an evanescent-field activated fluorescence detection principle (described later in greater detail).

2.2 Microarray Plate: LecChip™

LecChip™ Ver.1.0 is a commercial product for the lectin microarray: it contains 45 different lectins (for a list see [5] or [37]), which were carefully selected from a pool of 167 candidate lectins, taking into consideration their binding specificity, stability, and economy. Lectins are briefly classified based on monosaccharide

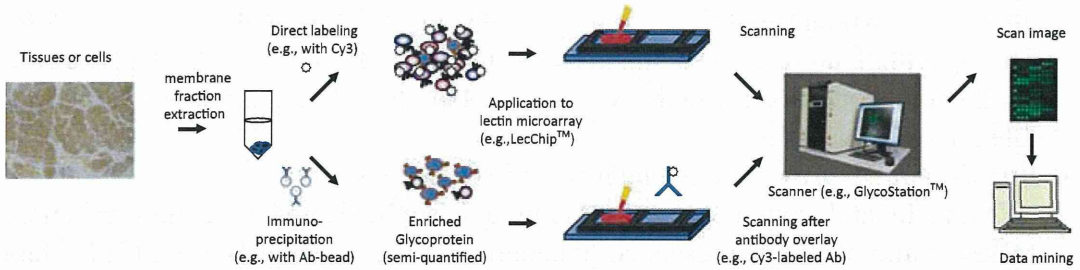

Fig. 1 General procedures of lectin microarray analysis: from sample preparation to data analysis. There are basically two schemes for glycan profiling; i.e., an *upper scheme* for direct profiling and a *lower scheme* for indirect profiling. In the former, all proteins prepared from cells and tissues are fluorescently labeled, e.g., Cy3, and their glycan profiles analyzed directly, while in the latter, target glycoproteins are immuno-precipitated prior to application to the microarray. The target glycoproteins are specifically detected by overlaying fluorescently-labeled detecting antibodies raised against the core protein moiety of the targets (for details, see text or Kuno et al. [28])

specificity; e.g., fucose binders (AAL, etc.), sialic acid binders (SSA, etc.), galactose binders (RCA120, etc.), mannose binders (ConA, etc.), and *O*-glycan binders (Jacalin, etc.), while some of them discriminate branching or more complex features (e.g., PHA-L). LecChip™ (ver.1.0) contains 7 wells on a glass slide, each containing the same set of 45 lectins spotted in triplicate. Basic information about these lectins is available on the website "Lectin frontier DataBase" (L*f*DB; http://riodb.ibase.aist.go.jp/rcmg/glycodb/LectinSearch), in which specificity data obtained by FAC [32, 33] are also available.

2.3 *Detection Principles*

1. *Confocal fluorescence detection method*

   Many researchers using the lectin microarray take advantage of a confocal fluorescence detection principle, because the method has already been adapted for established systems of DNA microarray [18, 19]. The method is simple, and has therefore been widely used. However, it requires washing of the microarray surface after the binding reaction, because a dried sample is required for the confocal-type detection system. One useful application of the system is multi-color detection using, e.g., Cy3 and Cy5, as described later.
2. *Evanescent-field activated fluorescence detection method*

   Kuno et al. developed a unique lectin microarray system, which utilizes an evanescent-field activated fluorescent detection principle. The evanescent wave is generated from the surface of the glass slide when an appropriate light originally introduced from both edges of the slide makes a total reflection [10]. Because the range of this wave is largely restricted to "near optic field (i.e., substantially <200 nm)," fluorescently labeled materials, such as Cy3-labeled glycoproteins, cannot be activated unless they form a significant complex on the array surface. As a result, the method requires no washing