Application of Lectin Microarrays for Biomarker Discovery

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Many proteins in living organisms are glycosylated. As their glycan patterns exhibit protein-, cell-, and tissue-specific heterogeneity, changes in the glycosylation levels could serve as useful indicators of various pathological and physiological states. Thus, the identification of glycoprotein biomarkers from specific changes in the glycan profiles of glycoproteins is a trending field. Lectin microarrays provide a new glycan analysis platform, which enables rapid and sensitive analysis of complex glycans without requiring the release of glycans from the protein. Recent developments in lectin microarray technology enable high-throughput analysis of glycans in complex biological samples. In this review, we will discuss the basic concepts and recent progress in lectin microarray technology, the application of lectin microarrays in biomarker discovery, and the challenges and future development of this technology. Given the tremendous technical advancements that have been made, lectin microarrays will become an indispensable tool for the discovery of glycoprotein biomarkers.

1. Introduction

A biomarker is a biological indicator that objectively measures and evaluates normal and pathological biological processes, or responses to therapy. Biomarkers can be used for the diagnosis and classification of diseases, efficacy monitoring, disease risk prediction, and screening of high-risk groups.[1] They can be divided into clinical, imaging, and biochemical and genetic markers.[2a,b] Proteins and their posttranslationally modified forms are involved in almost all biological processes including gene expression regulation, cytokine formation, material transport, and metabolism. Thus, proteins and their modifications can potentially serve as objective indicators to evaluate normal physiological function or pathological status.

Glycosylation is the most common posttranslational modification, wherein over half of proteins are glycosylated.[3] Glycosylation plays essential roles in various biological processes such as cell proliferation and differentiation, intercellular communication and signaling, cell–cell and protein–protein interactions, cell adhesion, and immune responses.[4a,b] Moreover, glycosylation patterns exhibit protein-, cell- and tissue-specific heterogeneity, thus enabling the assessment of changes in various pathological and physiological states such as tissue origin, tissue development, tumorigenesis, and the degree of malignancy.[5] For instance, the core fucosylation of α-Fetoprotein L3 is a common diagnostic glycoprotein biomarker for hepatocarcinoma; and the glycan antigen sialyl-Lea is a common marker for gastrointestinal cancer.[6a,b,c] Additionally, many tumors have been reported to show significantly increased expression of truncated O-glycans and N-Acetyl-D-glucosamine-branching N-glycan.[7a,b] Therefore, glycemic profiling of tissues under different physio-pathological states may contribute to the discovery of biomarkers that are associated with tissue- or disease-specific alterations. Over the past decade, mass spectrometry (MS)-based methods have been traditionally used for glycomic analysis.[8] Although MS-based methods can reliably identify the structure, linkage, and position of glycans, enzymatic or chemical stripping of glycans from proteins prior to MS profiling prevents accurate detection and identification of total glycans. Moreover, these methods are usually time-consuming and require complex sample preparation procedures.[9a,b]

A technique called lectin microarray, which was developed in 2005,[10a,b,c,d] has gained increasing popularity for high-throughput analysis of glycans. Lectins are a group of carbohydrate-binding proteins that specifically bind different glycans. The advantages of using lectin microarray over traditional MS-based methods include the simplicity and high sensitivity of the method that supports direct global glycomic profiling, the lower stringency of initial sample purity (crude glycoprotein samples can be analyzed), and the comparatively simple sample preparation procedure (without glycan-release and purification).[10a,b,c,d] As protein fragmentation or glycan liberation is not required during sample preparation, the sampled glycoproteins can retain their intact natural conformations and abundance. Thus, lectin microarrays are suitable for analyzing the differential glycomic profiles of biological samples. However, this technique is not quantitative and does not allow complete determination of glycan structures like MS. Instead, lectin microarrays are more appropriate for comparative purposes, such as for analyzing differences between glycomic profiles.[11] The sensitivity, simplicity, and robustness of lectin microarrays require further improvement to broaden their applications.

Currently, lectin microarray is widely used to assess tumor characteristics and to screen for novel diagnostic cancer biomarkers.[12] In this paper, we will review the recent advances in lectin microarray technology and focus on the recent progress and application of lectin microarrays in biomarker discovery under various pathological and physiological states.
2. Lectin microarray strategies for biomarker discovery

2.1. Direct Assay

Lectins with known specificity were first immobilized through either covalent bonding or physical adsorption before incubation with the fluorescently-tagged samples. Subsequently, the binding event can be monitored through fluorescence detection (Figure 1A). This assay can be used to analyze differential glycosylation patterns of normal versus disease samples or to investigate the effect of various treatment conditions. Traditional lectin arrays can only show the bulk glycosylation levels of abundant proteins, which have a relatively higher representation in the protein mixture. To minimize the bias towards identification of abundant proteins, Etxebarria et al. developed a fluorescence-based method for the rapid analysis of protein glycosylation in biofluids. In this method, fluorescently-tagged glycoproteins that have been transferred to a lectin-coated slide retain their relative positions on the SDS-PAGE gel. The individual lectin binding profiles for all separated proteins, independent of abundance, can thus be obtained.

2.2. Lectin-Overlay Antibody Sandwich Array

This method developed by Chen et al. involves the use of antibody microarray capture of multiple proteins followed by detection using various biotinylated lectin probes (Figure 1B). Chemical derivatization of glycans on captured antibodies is an essential preliminary step in this method to prevent the binding of lectin probes to the glycans. Derivatized antibodies are immobilized on nitrocellulose slides, and unprocessed samples can be directly loaded onto the array. Chen et al. successfully identified cancer-associated glycan alterations on MUC1 and CEA proteins in the serum of pancreatic cancer patients using this method. Thus, this method is thought to be particularly useful for profiling variations of specific glycans on multiple proteins. To measure the amount of both glycans and glycoproteins in a crude sample, Yue et al. used a dual antibody sandwich assay to determine protein levels and a lectin-overlay antibody sandwich array to characterize glycan levels. This method may potentially be useful for characterizing the rate of alterations to the glycans and the relationship between glycans and their carrier proteins.

2.3. Antibody-Overlay Lectin Sandwich Array

This assay was initially developed by Rosenfeld et al. in 2007. As a brief summary, the lectins spotted on nitrocellulose membrane-coated glass slides are used to capture the target glycoproteins. The selectively-bound glycoproteins are then

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probed with either directly labeled or indirectly labeled secondary antibodies. The arrays are scanned and analyzed to generate a characteristic fingerprint that mirrors alterations in the protein’s glycan composition (Figure 1C). Rosenfeld et al. developed Qproteome™ GlycoArray kits based on this method, which enables rapid analysis (within 4–6 hours) of the glycosylation profiles of intact glycoproteins. Kuno et al. established antibody-assisted lectin profiling to detect glycosylation changes in low-abundance target molecules. This method involves enrichment of target proteins with specific antibodies by immunoprecipitation followed by quantification with immunoblotting. An accurate glycan profile of the target glycoprotein can be obtained at the sub-picomolar level from a trace amount of crude samples in a highly reproducible and high-throughput manner. Although weak lectin–glycan interactions can be detected using this method, a specialized evanescent-field fluorescence scanner is necessary to increase sensitivity. An improved version of the antibody-overlay lectin microarray method involving the integration of tyramide signal amplification (TSA), a horseradish peroxidase (HRP)-mediated signal amplification method, was developed by Meany et al. to further boost sensitivity by over 100 times. In addition, this improved method does not require specialized instruments for detecting weak lectin–glycan interactions. Thus, this enables glycan profiling at the sub-nanogram level.

2.4. Glycoprotein–Lectin Array

In this reversed-phase lectin microarray, glycoproteins are first enriched using a general lectin column and are then separated by reversed-phase high-performance liquid chromatography (HPLC). The purified glycoproteins are then immobilized on the slide surface and probed with labeled lectins with a wide range of glycan binding specificities (Figure 1D). This method enables the profiling of glycan distribution in the human glycoproteome and the monitoring of individual glycosylation alterations at a global scale. This array could successfully identify serum biomarkers in pancreatic diseases. However, the high effective sample concentration on one spot can increase the risk of spurious interactions.

3. Factors Influencing Lectin Microarrays for Biomarker Discovery

3.1. Types of Lectins

3.1.1. Natural Lectins

Natural lectins are lectins that are purified from plants, animals, bacteria, or fungi. Among them, plant lectins, also known as (phyto)hemagglutinins, are the most widely used lectins for glycan profiling due to their availability, stability, and varied binding specificities. For example, the families of B-chain-like lectins, legume lectins and ricin, can bind to a wide range of glycans. The jacalin-related lectins are both Man- and Gal-specific, but monocot-derived lectins are only specific for Man and hevein-type lectins only for GlcNAc. Disadvantages of natural lectins include inherent glycosylation, batch-to-batch variation, and inconsistencies in activity due to differences arising from purification procedures. Nonetheless, advancements in genome technology as well as glycotechnology are expected to support the development of more useful natural lectins with unique glycan-binding specificities. The origin, monosaccharide specificity, and preferred
glycan structure as well as specificity profile of natural lectins mentioned in this review are summarized in Table 1.

### 3.1.2 Recombinant Lectins

Recombinant lectins, particularly those of plant and animal origin, are produced from microorganism expression systems by recombinant technology. Bacteria and yeasts, which have simple gene expression systems and good recombinant protein yield, are the preferred tools for recombinant lectin generation. As recombinant lectins can overcome the limitations of natural lectins, they have been increasingly used for glycan profiling. The source organism, organism used for clonal expansion, and specificity profile of recombinant lectins mentioned in this review are summarized in Table 2.

Hsu et al. was the first to develop the protocol and demonstrate the use of recombinant lectins in microarrays. They expressed bacteria-derived lectins in Escherichia coli as fusion proteins and then purified them using glutathione (GSH) affinity chromatography. Lectin activity and glycan-binding specificities (glycopatterns) of the purified lectins for both proteins and cell samples could be determined using carbohydrate microarray and ELISA. Increasing the accessibility of the carbohydrate-binding site can enhance the sensitivity of the

| Table 1. Specificity profile of natural lectins. |
|---|
| **Lectin name** | **Origin** | **Monosaccharide specificity** | **Preferred glycan structure** | **Refs.** |
| **Fungal lectins** |  |
| AAL | *Aleuria aurantia* | Fuc | Fuc(α6GlcNAc (core Fuc), Fuc(α3(Galβ4) GlcNAc (Le*) | [36] |
| ABA | *Agaricus bisporus* | Gal, GlcNAc | Gal(β3GalNAc, GlcNAc | [36] |
| ACG | *Acrogybe cylindracea* | Gal | Siaβ3Galβ4GlcNAc | [31] |
| **Plant lectins** |  |
| ACA | *Amaranthus caudatus* | Gal | Galβ3GalNAc | [32] |
| ACL | *Amaranthus caudatus* | Gal | Galβ1-3GalNAc | [33] |
| BPL | *Bauhinia purpurea* | Gal | Galβ3GalNAc, GalNAc | [34] |
| BS-I | *Bandiera simplicifolia* | Gal, GalNAc | αGal, αGalNAc | [28a] |
| ConA | *Cannavalia ensiformis* | Man | High-Man including Manα6(Manα3) Man | [35] |
| DBA | *Dolichos biflorus* | GalNAc | Gal(β3GalNAc | [36] |
| DSA | *Datura stramonium* | GlcNAc | (GlcNAcβ4) triantennary, tetraantennary N-glycans | [37] |
| ECA | *Erythrina cristagalli* | Gal | Galβ4GlcNAc | [38] |
| GNA | *Gallus gallus* | N-glycans | High-Man including Manα3Man | [39] |
| GSL–I–B4 | *Griffonia simplicifolia* | Gal | αGal | [34] |
| GSL–II | *Griffonia simplicifolia* | GlcNAc | Agalactosylated tri/tetra antenney glycans, GlcNAc | [40] |
| HHL | *Hippocastanum Hybrid* | Man | High-Man including Manα3Man or Manα6Man | [41] |
| Jactalin | *Arctocarpus integrifolia* | Gal | Galβ3GlcNAc, αGalNAc (6-O-unsubstituted) | [42] |
| LCA | *Lens culinaris* | Fuc/Man | Fucα6GlcNAc, High-Man | [40] |
| LEL | *Lycopersicon esculentum* | GlcNAc | (GlcNAcβ4)α(Galβ4GlcNAc) α(polylactosamine) | [44] |
| LTL | *Lotus tetragonolobus* | Fuc | Fucβ3Galβ4GlcNAc (Le*), Fucβ2Galβ4GlcNAc (H-type 2) | [45] |
| MPL | *Maackia amurensis* | GalNAc | αGalNAc | [28a] |
| NPA | *Narcissus pseudonarcissus* | Man | High-Man including Manα3Man | [41] |
| PHA-En | *Phaseolus vulgaris* | Gal | N-glycans with outer Gal and bisecting GlcNAc | [46] |
| PHA-L | *Phaseolus vulgaris* | Complex | Tri/tetra-antennary complex-type N-glycan | [46] |
| PNA | *Arachis hypogaea* | Gal | Galβ3GlcNAc | [34] |
| PSA | *Pisum sativum* | Fuc/Man | Fucα6GlcNAc, High-Man | [42] |
| PTL-I | *Pspoposcarus tetragonolobus* | GalNAc | αGalNAc | [47] |
| PWM | *Phytolacca americana* | GlcNAc | (GlcNAcβ4)αGalNAc | [48] |
| RCA-I | *Ricinus communis* | Gal | Galβ4GlcNAc | [36] |
| SBA | *Dolichos biflorus* | GalNAc | GalNAcβ3GalNAc | [34] |
| SNA | *Sambucus nigra* | Sia | Siaβ2-6GalβGalNAc | [36] |
| SSA | *Sambucus sieboldiana* | Sia | Siaβ2-6GalβGalNAc | [40] |
| STL | *Solanum tuberosum* | GlcNAc | (GlcNAcβ3)αGlcNAcβ4MurNAcα (peptidoglycan backbone) | [51] |
| TJA-I | *Tanthes japonica* | Sia | Siaβ2-6GalβGalNAc | [36] |
| UDA | *Uricia dioica* | GlcNAc | GlcNAcβ4Manβ3Manβ5-Man9 | [32] |
| UEa-I | *Ulex europaeus* | Fuc | Fucβ2Galβ4GlcNAc (H-type 2) | [32] |
| VVA | *Vicia villosa* | GalNAc | αGalNAc, GalNAcβ3Gal | [34] |
| WFA | *Wisteria floribunda* | GalNAcβ3GlcNAc, Galβ3(6-b) GalNAc | [34] |

| a) Fuc, fucose; Gal, galactose; GlcNAc, N-acetyl-D-glucosamine; Man, mannose; GalNAc, N-Acetyl-D-galactosamine; Sia, sialic acid |

| Table 2. Specificity profile of recombinant lectins. |
|---|
| **Lectin name** | **Source organism** | **Organism used for clonal expansion** | **Specificity Refs.** |
| **MBL** | *Gallus Gallus* | *Helix R19 Cells* | | [54] |
| GaFD | *Escherichia coli* | *Escherichia coli* | | [57a, 58] |
| PA-IL | *Pseudomonas aeruginosa* | *Escherichia coli* | | [57a, 58] |
| PA-III | *Pseudomonas aeruginosa* | *Escherichia coli* | | [57a, 58] |
| PapGIII | *Escherichia coli* | *Escherichia coli* | | [57a, 58] |
| PapGII | *Escherichia coli* | *Escherichia coli* | | [57a, 58] |
| RS-II | *Ratstonia solanacearum* | *Escherichia coli* | | [57a, 58] |
| Mutated MAH | *Masckia amurenis* | *Escherichia coli* | | | [52] |
| EW99 | *Earthworm* | *Escherichia coli* | | [52] |
| EW29Ch | *Earthworm* | *Escherichia coli* | | [52] |
3.2. Detection Techniques

3.2.1. Label-Based Techniques

Fluorescence, chemiluminescence, and radioactivity are three popular label-based techniques for detecting a target amidst a complex background via direct or indirect labeling. The advantages of this method are that it is conveniently applied with commonly available reagents and uses a simple experimental procedure. Fluorescent labels, such as Cyanine 3 (Cy3) or 5 (Cy5), are commonly used in lectin microarray detection.

Direct labeling is the most common method for identifying lectin–glycan interactions. Lectins or glycoproteins in samples, such as formalin-fixed paraffin-embedded tumor tissues, serum, and urine, are labeled directly with a fluorescent dye and are subsequently washed and detected with a fluorescence scanner. Although direct labeling is often the preferred labeling method, the drawbacks include the requirement for a relatively high amount of glycoproteins, low sensitivity, and a potential disruption of interactions between glycoproteins and lectins.

The single-color lectin microarray is a direct labeling method that has been established to study the glycoprofiling of mammalian cells. This method has multiple flaws in the protocol, such as no reliable quality control, poor reproducibility, and disregard of the effects of cellular glycolipids. Pilobello et al. then developed a two-color lectin microarray approach that can rapidly determine the difference in glycoprofiling among heterogeneous mammalian samples. Either Cy3–NH$_3$ or Cy5–NH$_3$ dye molecules are conjugated with the lysines within proteins in this two-color direct labeling method. The Cy3- and Cy5-labeled samples are mixed in a 1:1 ratio and are hybridized to each lectin microarray. This method may be applied for the systematic evaluation of glycan information in complex systems. However, careful consideration should be exercised during the quantitative comparison of lectin signals for the two-color labeling method due to potential competition between immobilized lectins for various glycans.

The indirect labeling method is generally used in the sandwich format in assays, wherein the lectin–glycoprotein interaction is detected using a biotinylated antibody and a corresponding fluorescent dye (HRP)-conjugated streptavidin. Meaney et al. included Cy3-labeled streptavidin into this system to increase the sensitivity of targeted glycan profiling. Cy3-labeled streptavidin further amplified the signal from biotin that have conjugated with HRP-conjugated streptavidin by over 100 times. Cao et al. introduced a lectin multimerization approach to increase lectin avidity to targeted glycans, wherein several biotinylated lectins are conjugated through streptavidin interactions. Proteins in biological samples are captured by immobilized antibodies on arrays, and the glycans on the captured proteins are probed with biotinylated lectins. Unlike the conventional non-multimerization method wherein primary and secondary detection reagents are added consecutively, these reagents are added in a single step for the multimerization method. Single-step addition may enable enhanced binding through multivalent interactions. Thus, the multimerization method can potentially broaden the range of glycan structures that can be detected and provide more and different information compared to monomeric detection. Because of low background labeling and signal amplification, the indirect labeling methods have advantages of high specificity and sensitivity.

Confocal fluorescence has been widely used for lectin microarray detection. However, the glycan–lectin interaction formed on the microarray may be disrupted due to the washing step after the binding reaction. Multicolor confocal fluorescence detection using, for example, Cy3 and Cy5 dyes can be used to analyze relatively weakly bound glycan on fluorescent dye-labeled glycoproteins. Kuno et al. introduced an evanescent-field fluorescence detection method for real-time detection of interactions under equilibrium conditions without washing on a lectin microarray after a probing reaction. Koshi et al. developed a bimolecular fluorescence quenching and recovery method for glycan detection in a lectin microarray. Fluorescently labeled lectins are non-covalently fixed under semi-wet conditions and recognize and bind to specific glycans. Labeling of target glycans is unnecessary because of high selectivity and affinity of glycans for the immobilized lectins.
3.2.2. Label-Free Techniques

Label-free techniques, such as surface plasmon resonance (SPR), optical microscopy, and MALDI-TOF MS, are used to determine the inherent properties (dielectric or optical properties and mass) of molecules bound to lectin microarrays. SPR, which monitors biomolecular interactions in real-time through measuring changes in the light reflected on the underside of the metal, is a common method for evaluating the affinity, kinetics, specificity, and concentration of biomolecules. Nand et al. coupled SPR imaging with a lectin microarray to discriminate between the different stem cells. Geuien et al. established a lectin microarray with multiplex SPR capable of accurately quantifying the relative sialylation levels of erythropoietin. Since optical methods are sensitive and non-destructive, they have been used to measure lectin–glycan interactions on a solid surface. Optical microscopy can be used to observe distinct binding patterns of glycans on a lectin array. Chen et al. used this approach to detect differences in glycoprofiles between normal and tumorogenic human breast cell lines, as well as determine the metastatic potential of different sublines. Unlike MS, a lectin microarray cannot provide in-depth structural information on glycans. However, combining lectin microarrays with MS enables high-throughput glycoprotein biomarker screening, accurate mass measurement, and resolution of glycoproteins that bind to each immobilized lectin on the array. Of note, MALDI-MS detection of captured proteins on the array allows quantitative measurement of glycosylation levels as well as detection of non-specific binding. Indeed, Hu et al. validated this method of coupling MALDI-TOF-MS with a lectin microarray on polydimethylsiloxane for the differential analysis of serum glycoproteins in oral cancer versus healthy control subjects.

4. Application of Lectin Microarrays for Biomarker Discovery

4.1. Cancer Biomarker Discovery

4.1.1. Lung Cancer

Lung cancer is the most common disease worldwide. Small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC) are the two common types. The subtypes of NSCLCs include lung adenocarcinoma (ADC), squamous cell lung cancer (SQC), and large-cell carcinoma. As current diagnostic methods such as computed tomography, X-ray, and sputum cytology are lacking in detecting early stages of lung cancer, it is thus important to elucidate serum biomarkers for early cancer detection, cancer staging determination, and for monitoring the response to therapeutic strategies. Although several serum proteins, including carcinoembryonic antigen, carbohydrate antigen 125, cytokeratin 19, and neuron-specific enolase, have been proposed as potential biomarkers of lung cancer, low specificity and sensitivity limit their clinical use. Glycan profiling may offer an alternative approach to identify specific biomarkers for lung cancer.

Shi et al. showed the distinct glycoprotein profiles of cells in pleural effusions from lung cancer patients (carcinoma cells) versus those with benign lung disease (reactive mesothelial cells) using lectin microarrays. Analysis of serum N- and O-glycan profiles in NSCLC patients versus healthy controls by Liang et al. using lectin microarrays revealed 18 lectins (e.g., Aleuria aurantia lectin [AAL], Jacalin, Griffoniasimplicifolia Lectin I [GSL–I], and Dolichos biflorus [DBA]) in lung adenocarcinoma and 16 lectins (e.g., Jacalin, Hippeastrum hybridelectin [HHL], Phaseolus vulgaris erythroglauculinin [PHA–E], and Phaseolus vulgaris leucoagglutinin [PHA–L]) in SCLC with significantly altered serum glycopatterns. Notably, the majority of lectins that showed altered expression profiles were found in patients with early stage adenocarcinoma and SCLC (Table 3).

Recent studies reported an increase of human α-1-antitrypsin (A1AT; a serum glycoprotein with three potential glycosylation sites) levels in various cancers including lung, prostate, and breast cancers. Elevated A1AT levels were also noted in certain benign pulmonary diseases. Liang et al. examined A1AT glycosylation alterations in ADC, SQC, and SCLC serum samples using lectin microarrays coupled with ELISA. Three markers were identified. Galactosylated A1AT was identified as a marker capable of differentiating NSCLC from benign pulmonary diseases; fucosylated A1AT as a marker to distinguish ADC from benign diseases or other lung cancer subtypes; and A1AT containing poly–LacNac as a marker to differentiate SCLC from benign diseases (Table 3). Due to the small sample size in the Liang et al. study (12 samples for each group), further validation of the potential role of glycosylated A1AT as a lung cancer biomarker is necessary using larger sample sizes.

Hira et al. developed an integrated glycoproteomics approach to identify a glycoprotein biomarker that is capable of differentiating NSCLC from SCLC. Tissue extracts and cell culture supernatants were added to the lectin microarray to identify NSCLC-specific lectin probes. Next, isotope-coded glycosylation site-specific tagging-LC-MS-based screening of glycobiomarker candidate molecules present in the supernatants from SCLC and NSCLC cell lines was performed. The preliminary candidates were then examined using a Western blot and immunoprecipitation analyses before final reevaluation using an antibody-overlay lectin microarray. A successful example of this strategy is the identification of NSCLC-specific biomarkers like fibronectin that have fucose- and oligomannose-modified N-glycans (Table 3).

4.1.2. Gastric cancer

Gastric cancer is the most common epithelial tumor worldwide and ranks second in cancer-related mortality rates. The relationship between stomach ulcers and cancer has long been controversial. Huang et al. observed higher glycosylation levels in gastric cancer tissues than in gastric ulcer tissues from a 37-lectin microarray. Notably, two lectins, Maclurapomifera
Table 3. Application of lectin microarrays for biomarker discovery of various diseases.

| Diseases                     | Sample type     | Glycoprotein marker | Specific lectin (target glycan) | Microarray Strategy | Potential applications                                                                 | Refs. |
|------------------------------|-----------------|---------------------|--------------------------------|---------------------|----------------------------------------------------------------------------------------|-------|
| Cancer                       | Serum           | Not identified      | AAL ([Fucα1-6GlcNAc]; Jacalin [Galα1-3GalNAcα]; GSL-1 [GlcNAc and αGal]; DBA [GalNAc and GalNAcα1-3Gal]; HHHL [High-Man, Manα1-3Man, Manα1-6Man, Man5-GlcNAc2]; PHA-E±L (the bisecting GlcNAc and biantennary N-glycans) | Direct assay | Early diagnosis of lung adenocarcinoma and squamous cell lung cancer                      | [95]  |
| Lung cancer                  | Serum           | α-1-antitrypsin     | BS-I (αGal and αGalNAC); AAL ([Fucα1-6GlcNAc and Fucα1-3Galβ1-4GlcNAc]; PWM (Branched [LacNAc]in) | Direct assay | Distinguish non-small-cell lung cancer, lung adenocarcinoma and small-cell lung cancer from benign pulmonary diseases | [96]  |
| Gastric cancer               | Tissues and cell culture supernatants | Fibronectin | PNA (Galβ1-3GalNAc) | Antibody-overlay lectin sandwich array | Biomarker of non-small-cell lung cancer | [97]  |
| Tissues                      | Serum           | Not identified      | MPL (GalNAc); VVA (GalNac and GalNAcct) | Direct assay | Distinguish gastric cancer from ulcer                                                   | [98]  |
| Tissues                      | Serum           | Not identified      | LEL and STL (GlcNAc) | Direct assay | Diagnostic marker for early gastric cancer                                              | [99]  |
| Colorectal cancer            | Plasma          | Complement C3; histidine-rich glycoprotein; kininogen-1 | AAL and SNA (sialylation and fucosylation) | Glycoprotein-lectin array | Distinguish colorectal cancer from adenoma and normal                                     | [100] |
| Tissues                      | Serum           | Complement C3       | HSP90βI and Annexin A1 | Direct assay | Diagnostic marker for colorectal cancer                                                 | [101] |
| Tissues                      | Serum           | Alpha-2-macroglobulin | No identified | ABA (Galβ1-3GalNAcct) | A predictive biomarker for recurrence of colorectal cancer                              | [102] |
| Cell lines                   | Serum           | Not identified      | UEA-1 (α-1,2-fucosylation) | Direct assay | Lectin biomarker for colorectal cancer                                                  | [103] |
| Hepatocellular carcinoma     | Cell lines      | Not identified      | ALC, BPL, IAC, MPL [Galβ1-3GalNAcct and GalNAcct]; PHA-E (NA2 and bisecting GlcNAc); SNA (Siaα2-6Gal/GalNAc); SBA (terminal α or βGalNAc) | Direct assay | Metastasis-specific glycan markers                                                      | [104] |
| Cell lines                   | Serum           | Immunoglobulin G    | PSA, LCA, and AAL (core α-1,6 fucosylation); SNA-I (α-2,8 sialylation) | Direct assay | Disease diagnosis                                                                        | [105] |
| Cell lines                   | Serum           | GP73                | AAL ([Fucα6GlcNAc (core Fucu), Fucα3Galβ1) 4GlcNAc(Lex); LCA (Fucα6GlcNAc, High-Man); PSA ([Fucα6GlcNAc, High-Man) | Antibody-overlay lectin sandwich array | Distinguish HCC from liver cirrhosis                                                   | [106] |
| Cell lines                   | Serum           | Not identified      | PHA-L (β1,6-GlcNAc) | Direct assay | Metastasis-related marker                                                               | [107] |
| Cell lines                   | Serum           | Not identified      | HHL and NPA (Man1-6Man) | Direct assay | Disease diagnosis                                                                        | [108] |
| Cell lines                   | Cell lines      | Annexin A2; Heat shock protein 90 | βeta family member | Direct assay | Diagnostic biomarkers for hepatoma cells after HCV infection                             | [109] |
| Cell lines                   | Cell lines      | POTE ankyrin domain family member F | RCA-1 (Lacα1LacNAc, Terminal Galβ1-4GlcNAcβ1) | Direct assay | Marker for metastatic triple-negative breast cancer cells                                | [110] |

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lectin (MPL) and Vicia villosa (VVA), have been identified and validated as specific gastric cancer biomarkers (Table 3).\[88\]

Shu et al. found 15 lectins (e.g., Pisum sativum [PSA], PHA–E, and Erythrina cristagalli [ECA]) that showed significantly altered salivary protein glycosylation in gastric cancer (adenocarcinoma of stage I/II/III) and atrophic gastritis patients versus healthy volunteers using lectin microarrays. Outer-arm fucosylation and core-fucosylation expression levels of glycans specific for AAL have been identified and validated as specific gastric cancer biomarkers (Table 3).\[88\]

| Diseases                  | Sample type       | Glycoprotein marker                  | Specific lectin (target glycan) | Microarray Strategy | Potential applications                                                                 | Refs. |
|---------------------------|-------------------|--------------------------------------|---------------------------------|---------------------|----------------------------------------------------------------------------------------|-------|
| Pancreatic cancer         | Cell lines        | Lysosome-associated membrane glyco- protein 1; hypoxia upregulated protein 1 | PSA (α1-3 core Fuc); DSA and PHAE (Galβ1-4GlcNAc; NPA, GNA, and HHL(High-Man) | Direct assay         | Disease diagnosis                                                                      | [106] |
|                            | Cell lines        | Cytokeratin 8, integrin β1, ICAM1, and ribophorin 2 CD133 | UEA-1 (Fuc); DBA (Gal)          | Direct assay         | Prognostic markers for pancreatic cancer                                                  | [103] |
|                            | Ascents fluids    | Not identified                       | SNA-1 (Galβ1-3Galβ1-4GlcNAc); SDL (Glcβ1-4GlcNAc); UDA (Glcβ1-4GlcNAc and Man) | Direct assay         | Prognostic biomarker for advanced pancreatic cancer                                      | [104] |
| Bladder cancer            | Cell lines        | Not identified                       | LT-1 (Galβ1 Lewis X); PTL-II (terminal GalNAc and Gal) | Direct assay         | Related to bladder cancer progress                                                      | [106] |
| Ovarian cancer            | Ascents fluids and culture supernatants of cell lines | Ceruloplasmin                      | WFA [Galβ1-4GlcNAc(LacdiNAc)] | Direct assay         | Disease diagnosis                                                                      | [106] |
| Inflammatory diseases     | Serum             | Matrix metalloproteinase-3           | ACG (Siaα2-3Galβ1-4GlcNAc); ABA (Galβ1-3GlcNAc); ACA (Galβ1-3GlcNAc) SNA-1 (Galα2-6 linked Sia) | Antibody-overlay      | Assess disease activity                                                                | [107] |
| Rheumatoid arthritis      | Serum             | Haptoglobin-related protein          | WFA[Galβ1-4GlcNAc, Galβ1-3Galβ1-4GlcNAc]; ABA and GSL-Ⅱ (agalactosyl N-linked oligosaccharides) | Direct assay         | Distinguish non-bacterial pneumonia from bacterial pneumonia                             | [108] |
| Pneumonia                 | Serum             | Mac-2 binding protein                | WFA(GlcnAcβ1-4GalNAc, Galβ1-3Galβ1-4GlcNAc) | Direct assay         | Related to liver fibrosis progression                                                  | [109] |
| Chronic hepatitis         | Serum             | Immunoglobulin G                    | ABA and GSL-Ⅱ (agalactosyl N-linked oligosaccharides) | Direct assay         | Correlated with disease activity and predictability of therapeutic outcomes            | [110] |
| Crohn's disease           | Serum             | Not identified                       | SSA (Siaα2-6Galβ1GlcNAc)        | Direct assay         | Predict the progression diabetic nephropathy                                            | [111] |
| Other diseases            | Urine             | Fetuin–A                            | TJA-Ⅰ, SNA, and SSA(Siaα2-6Galβ1GlcNAc) | Direct assay         | Clinical diagnosis and monitoring                                                       | [112] |
| Diabetic nephropathy       | Plasma            | No identified                        | ECA (Galβ1-4GlcNAc)            | Direct assay         | Characterized as human longevity and healthy aging                                      | [113] |
| Major depressive disorder  | Plasma            | Haptoglobin                         | ECA (Galβ1-4GlcNAc)            | Direct assay         | Clinical diagnosis of subfertility                                                      | [114] |
| Aging                     |                   |                                      |                                 |                     |                                                                                         |       |
| Subfertility              | Sperm             | Not identified                       | ABA (Galβ1-3GlcNAc); MPL (Galβ1-3GlcNAc and GalNAc) | Direct assay         |                                                                                         |       |

a) NeuAc, N-acetylgalactosamine; LacNAc, N-Acetyl-D-lactosamine; Lac, lactose

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4.1.3. Colorectal Cancer

Colorectal cancer is the third most common cancer worldwide.\[115\] The most common serum glycoprotein biomarker for colorectal cancer detection, carcinoembryonic antigen (CEA), has poor sensitivity and specificity and cannot be used for early cancer detection.\[116\] Similarly, other potential colorectal cancer serum glycoprotein markers, such as CA 19-9, CA 242, CA-195, CA 50, CA 74-2, and tissue metalloproteinase...
inhibitor\textsuperscript{1}\textsuperscript{,}\textsuperscript{120a,b,c,d} also have low sensitivity and specificity and are inadequate for screening or diagnostic applications.

Qi et al. developed lectin glycoarrays capable of identifying plasma markers from normal, adenoma, and colorectal cancer patients. Abundant plasma proteins are first subjected to immunodepletion, and then plasma N-linked glycoproteins are enriched using lectin affinity chromatography and nonporous silica reversed-phase HPLC. The enriched glycoproteins are then added to lectin microarrays to determine glycopolymers. Notably, complement C3, histidine-rich glycoprotein, and kinogen-1, which showed elevated sialylation and fucosylation levels, were identified as potential colorectal cancer markers (Table 3).\textsuperscript{96}

Li et al. established an integrated approach involving lectin microarrays and MS quantification for identifying candidate colon cancer biomarkers. Candidates with differential glycan profiles in colon cancer tissues versus adjacent normal colon tissues were first identified using a lectin microarray, and the shortlisted proteins are verified with lectin histochemistry. Next, enrichment and identification of specific lectins were performed using label-free MS. HSP90b and Annexin A1 proteins were found to be GlcNAcylated and their expression levels were upregulated in colon cancer tissues (Table 3).\textsuperscript{91}

Distant recurrence markers from formalin-fixed, paraffin-embedded tumor specimens and normal epithelium from 53 consecutive curatively resected stage I–III colorectal cancer patients (identified using lectin microarray in a study by Nakajima et al.) were validated with an additional 55 curatively resected stage II colorectal cancer cases. Notably, Agaricus bisporus (ABA) lectin, with high lectin–glycan interaction (LGI) values in cancer tissues and significant statistical association with distant recurrence, has been proposed as a novel biomarker for distant recurrence of curatively resected colorectal cancer (Table 3).\textsuperscript{92}

Sunderi et al. presented a lectin-based protein microarray to distinguish changes in alpha-2-macroglobulin (α2M) sera glycosylation levels between healthy individuals versus colorectal cancer patients. Target proteins are isolated through immunoprecipitation and then spotted onto the lectin microarray. The lectin-based protein microarray revealed a higher content of α2,6 sialic acid, N-acetylglucosamine and Man residues, and tri-/tetraantennary complex type high-Man N-glycans for α2M molecules isolated from the sera of colorectal cancer patients. Thus, the α2M glycopattern may be a potential colorectal cancer biomarker (Table 3).\textsuperscript{93}

The polyacrylamide hydrogel-based lectin microarray introduced by Tian et al. can be used to screen for colorectal cancer cells that express high levels of surface glycans. Multivalent lectins that are immobilized on the polyacrylamide hydrogel bind to the glycans with increased binding affinity and selectivity. Differentially expressed glycans with D-Gal, D-glucose, and sialic acid residues, and Ulex Europaeus Agglutinin–I (UEA–I) in SW480 colorectal cancer cells were identified from a 27-lectin-microarray screening. Further, in vitro and in vivo experiments confirmed UEA–I as a colorectal cancer biomarker (Table 3).\textsuperscript{94}

4.1.4. Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is the most prevalent primary liver cancer and ranks third in cancer-related deaths worldwide. HCC usually occurs in patients with an underlying chronic liver disease, such as cirrhosis or a chronic hepatitis B virus (HBV) infection.\textsuperscript{121} Changes in glycosylation levels are known to occur during HCC development.\textsuperscript{122}

Li et al. conducted glycoprofiling of intact Huh7 HCC cell surface glycoproteins in the epithelial mesenchymal transition (EMT) model using a lectin microarray to identify HCC metastasis-specific glycans. Decreased levels of T/Tn-antigen, NA2, bisecting GlcNAc, Siaα2-6Gal/GalNAc, terminal α1, or βGalNAc structures, and increased levels of terminal α1Fuc and α1,3Sia-Le, α1 or β-linked GalNAc, core fucose, β1,6 GlcNAc branching structures, and tetraantennary complex oligosaccharides were noted (Table 3).\textsuperscript{90}

Wang et al. developed a multiplex assay to analyze the glycopatterns of HCC-associated immunoglobulin G (IgG) and found increased (α1,6) fucosylation and (α1,2) sialylation levels in the IgG from the sera of HCC patients (Table 3).\textsuperscript{123} In this assay, a double-laser fluorescence system is used to identify biotin-labeled glycoproteins that bind to the immobilized lectins that are conjugated to fluorescent dye-coated microbeads. This system enables the three-dimensional interaction between lectins and specific glycans as well as the simultaneous detection of multiple glycan epitopes in a single reaction vessel.

Jiang et al. identified serum GP73, a resident Golgi type II membrane protein with three potential N-glycosylation sites, as a HCC diagnostic biomarker using antibody-overlay lectin microarray and a lectin blot. They found significantly higher fucosylated GP73 levels in liver cirrhosis patients compared to that in HCC patients. In addition, they showed that the combined detection of fucosylated GP73 and α1-fetoprotein-L3 increased the sensitivity and specificity of a HCC diagnosis (Table 3).\textsuperscript{97}

Liu et al. performed a lectin microarray, lectin affinity chromatography, and MS to elucidate biomarkers for diagnosing early hepatic encephalopathy (HE) in patients with terminal HCC using serum from HCC patients with or without early HE. As PHA–E levels showed a significant decrease in HCC patients with early HE, they concluded that 26 PHA–E-associated glycoproteins might be involved in the occurrence of early HE.\textsuperscript{122} In another study by Liu et al., 11 PHA–L reactive glycoproteins with a significantly altered N-glycosite occupancy (β1,6-GlcNAc branched N-glycan) were identified in HCC patients with metastasis, using a lectin microarray, lectin affinity chromatography, and stable isotope labeling coupled with LC-MS, which suggested their involvement in HCC metastasis (Table 3).\textsuperscript{98}

HBV and hepatitis C viral (HCV) infection-induced liver diseases are closely related to HCC. Analysis of the differential expression profiles of liver glycoproteins in normal pericardial tissues, liver cirrhosis, and tumor tissues induced by HBV using a lectin microarray revealed increased high-Man type glycans during cirrhosis-tumor progression in normal pericardial tissues, increased Manα1-3Man (GNA) in cirrhotic and tumor tissue, and
increased Man1-6Man (HHL and Narcissus pseudonarcissus [NPA]) only in tumor tissue (Table 3).\textsuperscript{109} Xiang et al. identified fucosylated annexin A2 and heat shock protein 90 beta family member 1, which showed significantly increased levels in HCV-infected Huh7.5.1 human liver cells, as potential markers using a combined lectin microarray with MS and a lectin pull-down assay (Table 3).\textsuperscript{100}

4.1.5. Breast Cancer

Arndt et al. showed that MDA-MB435 breast cancer cells bind to lectin ECA, Limax flavus (LFA) and Canavaliaensiformis (ConA) using a lectin microarray.\textsuperscript{124} Similarly, the 91-lectin microarray screening by Zhou et al. revealed that increased binding of Ricinus communis agglutinin I (RCA–I) to triple-negative breast cancer (TNBC) cells increases TNBC metastatic capacity. In addition, they found a differential galactosylation expression pattern of POTE ankynin domain family member F, which is capable of binding RCA–I, in high/low metastatic TNBC cells (Table 3).\textsuperscript{101}

Fry et al. found 6 lectins (GSL–II, Phytolaccacamerica [PWM], PNA, Psophocharpotetragonolobus [PTL–I], GSL–I–B₄, and jacalin) as potential metastatic primary breast tumor biomarkers using 45-lectin N- and O-linked glycan-specific microarrays coupled with evanescent-field activated fluorescence detection, glycomic analysis of primary breast tumors, and the serum and urine of patients with metastatic breast cancer. Increased binding to Aspergillus oryzae 1-fucose-specific lectin (AOL) and Galanthusnivalis agglutinin (GNA) lectins but decreased binding to Ricinus communis (RCA120) and PHA–E lectins were noted for the sera of metastatic patients. Three lectins, Trichosanthes japonica agglutinin I (TJA–I), RCA120, and Bauhinia purpurea lectin (BPL), displayed significantly increased levels in the urine of metastatic patients.\textsuperscript{88,104}

Guo et al. showed that conditioned medium (CM) derived from malignant breast cancer cells (MDA-MB-231 and MDA-MB-453) exhibit an altered N-glycan profile and induced an EMT-like process in non-tumorigenic normal mammary epithelial cells (MCF10A).\textsuperscript{125} Moreover, reduced levels of bisecting GlcNAc proteins, and the corresponding MGAT3 glycosyltransferase were observed in a hypoxia-induced EMT model using MCF7 and MDA-MB-231 breast cancer cell lines.\textsuperscript{126}

4.1.6. Pancreatic Cancer

Using a lectin microarray and LC-MS/MS, Tian et al. showed that the sialoglycoproteins LAMPI and ORP150 were overexpressed in the SW1990 human pancreatic cancer line (Table 3).\textsuperscript{102} Using the same strategy, Zhu et al. identified differentially expressed glycoproteins in pancreatic cancer CD24 + CD44 + stem-like cells and observed significantly increased fucosylated and galactosylated glycoproteins, such as cytokeratin 8/CK8, integrin β1/CD29, ICAM1/CD54, and ribophorin 2/RPN2, in CD24 + CD44 + cells (Table 3).\textsuperscript{103} Resistance to current pancreatic cancer therapies is heavily attributed to cancer stem cells (CSC). Terao et al. showed that fucosylation, a common modification in pancreatic cancer CSC-like cells from lectin microarray analysis, could serve as a novel biomarker to determine anticancer drug resistance.\textsuperscript{127} A lectin microarray performed by Sakae et al. revealed that the CSC marker CD133 in ascites-derived exosomes from patients with unresectable pancreatic cancer is commonly glycosylated by sialic acids. Thus, sialylated CD133 could potentially serve as a prognostic biomarker for advanced pancreatic cancer (Table 3).\textsuperscript{110}

4.1.7. Bladder Cancer

Using combined lectin microarray analysis, MALDI-TOF-MS and glycogene microarray analysis, Guo et al. showed decreased biantennary N-glycan structures and tetra-antennary complex-type N-glycan (recognized by PHA–E + L) levels during transforming growth factor-beta (TGF–β)-induced EMT in non-malignant bladder transitional epithelium HCV29 cells. This led to their conclusion of the involvement of α-mannosidase 2 and Type 1 α-L-fucosidase in TGF–β-induced EMT.\textsuperscript{128} Integrating lectin microarray and MS methods, Yang et al. found highly expressed core-fucosylated N-glycans but lowly expressed terminally fucosylated N-glycans in four bladder cancer cell lines (KK47, YTS1, J82, and T24) versus a normal bladder mucosa cell line (HCV29), suggesting their direct correlation with bladder cancer progression (Table 3).\textsuperscript{105}

4.1.8. Ovarian Cancer

Epithelial ovarian cancer (EOC), which accounts for 90% of all ovarian cancers, is usually asymptomatic and has a poor prognosis. The conventional marker CA125 often outputs false-negative results. Sogabe et al. discovered glycobiomarker candidates using a lectin microarray coupled with IOTG-LC/MS analysis. The cancer-associated glycopeptides are first enriched from ascites and culture supernatants of cancer cell lines using the lectin AAL before subsequent identification by IOTG-LC/MS. The EOC-specific lectin Wisteria floribunda agglutinin (WFA) was used for subsequent Western blot analysis to elucidate glycomembrin candidates from the WFA-bound fraction of ascites fluids. The WFA-reactive ceruloplasmin generated higher signals in the ascites fluids of EOC patients (Table 3).\textsuperscript{106} Zhao et al. performed lectin microarray-MS glycomic analysis of ovarian cancer side population cells to determine antigens associated with cancer recurrence and drug resistance. They observed increased core fucosylated N-glycan and tumor-associated Tn, T, and st antigen levels but decreased hybrid glycan, α2,3-linked sialic glycan, and multivalent sialyl–glycan levels in side population cells.\textsuperscript{129}

4.2. Inflammatory Diseases Biomarker Discovery

The common serological biomarker for rheumatoid arthritis matrix metalloproteinase-3 (MMP-3) lacks specificity and accu-
racy. An antibody-overlay lectin microarray of MMP-3, immuno-
precipitated from the sera of rheumatoid arthritis patients, led
to the identification of altered glycoprofiles of sialogic acid-
binding lectin ACG and O-glycan-binding lectins (Jcacin, ABA,
and *Amaranthus caudatus* lectin agglutinin [ACA]). Thus, changes
in MMP-3 glycosylation levels could serve as a potential rheuma-
toid arthritis-specific biomarker (*Table 3*).130

Yang et al. found increased lectin SNA–I signal in the
mycoplasma and viral pneumonia groups using lectin micro-
array coupled with LC-MS/MS. They identified haptoglobin-
related protein (HPR) from serum samples of patients with
mycoplasma pneumonia by a SNA–I pull-down assay and
further confirmed elevated SNA–I expression in the mycoplas-
ma pneumonia and viral pneumonia groups versus the bacterial
pneumonia group (*Table 3*).131

The serum levels of Mac-2 binding protein (M2BP) and
M2BPGi, a specific glycoform recognized by WFA, are positively
correlated with liver fibrosis progression. Narimatsu et al. found
that WFA lectin exhibits significantly high specificity for M2BP
but did not bind to most serum proteins in normal serum
samples using a lectin microarray. The M2BPGi assay kit was
then established and was validated using more than 8,000
samples. Thus, M2BPGi levels are an excellent diagnostic marker
for chronic hepatitis and cirrhosis (*Table 3*).132

Human inflammatory bowel disease (IBD), Crohn’s disease
(CD), and ulcerative colitis (UC) are characterized by chronic
recurrence and remission of digestive tract inflammation.
Significantly higher agalactosyl fraction among fucosylated
oligosaccharides of serum IgG was reported in CD and UC
patients. A lectin microarray screening revealed that ABA and
GSL–II lectins exhibit higher affinity for serum agalactosyl IgG
from IBD and especially CD patients. The observation of higher
agalactosyl IgG levels in CD patients was confirmed with a ABA
or GSL–II lectin–enzyme immunoassay. Thus, agalactosyl IgG
levels could serve as a novel biomarker for IBD (*Table 3*).133

### 4.3. Other Diseases Biomarker Discovery

Diabetic nephropathy (DN), a serious complication of diabetes,
is the main cause of chronic and terminal kidney disease
worldwide. Glycoprofile alterations in the urine can be used to
predict and monitor DN. A lectin microarray of urine samples
from DN patients revealed increased Siaα2-6Gal/GalNAc-bind-
ing lectin (SNA, SSA, TJA–I) signals in the urine samples of DN
patients. Fetuin-A glycoprotein was identified as the prognostic
biomarker for DN progression (*Table 3*).134 Another study
confirmed the positive correlation between Siaα2-6Gal/GalNAc
(recognized by SNA lectin) expression levels and DN progres-
sion and suggested its application in differentiating DN from
nondiabetic renal disease (NDRD).135 However, Yang et al.
showed that another glycan, (β1-4A)-linked GlcNAc, recognized
by the lectin *Datura stramonium agglutinin* (DSA), could be a
biomarker capable of differentiating DN from NDRD.136 Thus,
recent research to confirm the candidates for DN biomarkers is
necessary to resolve these inconsistencies.

Yamagata et al. noted common changes in Siaα2-6Gal/
GalNAc glycan in both depression mice models and major
depressive disorder (MDD) patients using a 45-lectin microarray.
Moreover, decreased ST6GALNA2 expression levels were
noted in the leukocytes from MDD patients. Thus, they
concluded that Siaα2-6GalNAc glycan in plasma protein and
ST6GALNA2 glycan in peripheral leukocytes may serve as
potential biomarkers for MDD clinical diagnosis and monitoring (*Table 3*).137

Glycans are emerging as aging biomarkers. A lectin micro-
array analysis showed the differential expression of α2-6sialy-
lated and α2-3sialylated O-glycan glycans during cellular
senescence between elderly- (86-year old and 97-year old
subjects) versus fetus-derived human skin fibroblast
samples. In contrast to these membrane glycoproteins that
decrease with age, α2-3/2-6sialylated intracellular glycoproteins,
except for some α2-3sialylated O-glycans, increase with age.109
Lectin microarrays coupled with LC-MS of plasma proteins from
Japanese semi-supercentenarians (106–109 years), aged
controls (70–88 years), and young controls (20–38 years) revealed
increased binding to ECA lectins. Abundant tri-antennary,
and sialylated N-glycans of haptoglobin at Asn207 and Asn211 sites,
which are abundant in semi-supercentenarians, are signatures
of extreme human longevity (*Table 3*).108

The glycocalyx coating on the sperm surface is vital for
sperm motility, maturation, and fertilization. Comparing the
binding abilities of multiple lectins in seminal plasma of fertile
men with that in infertile men, using a lectin microarray,
revealed that lectin reactivity is positively associated with fertility.109
β-defensin 126 (DEFB126) contributes to sialylation on the
sperm surface. Homozygous DEFB126 mutations can
lead to male subfertility as evident from decreased binding
affinity for 6 lectins (Jcacin/AIA, *Gossypium hirsutum agglutinin*
[GHA], *Amaranthus caudatus* lectin [ACL], *Maclurapomifera* lectin
[MPL], *Vicia villosa* lectin [VVL], and ABA). Of the 6 lectins, ABA
and MPL lectins were validated as potential DEFB126 homo-
ygous mutant male subfertility biomarkers (*Table 3*).110

### 4.4. Stem Cells Biomarker Discovery

Since glycan profiles undergo characteristic changes during
development, they are used as stem/progenitor cell markers.
Glycoprofiling of three different pluripotent stem cells
(mouse embryonic stem cells [ESCs], mouse-induced pluripotent
stem cells [iPSCs], and mouse embryonic fibroblast stem cells
[MEFs]) versus non-pluripotent cells elucidated 8 lectins (DBA,
*Maackia amurensis* [MAL], PHA-E, PHA-L, AAL, PNA, and
*Sambucus nigra* [SNA]) as potential pluripotency markers for
MEFs111 and 3 lectins (EEL, MAL and PHA–L) as differentiation
potency markers for human ESCs and iPSCs.111 Tateno et al.
developed a high-density 96-lectin microarray with broader
glycome coverage and found *Burkholderia cenocepacia* (rBC2LCN)
can distinguish undifferentiated iPSCs/ESCs from
differentiated SCs.112 Podacolyxin, a heavily glycosylated type
transmembrane protein, was identified as a glycoprotein ligand
of rBC2LCN on human iPSCs and ESCs using an antibody-
overlay lectin microarray. Moreover, significant affinity of rBC2LCN for a branched O-glycan with a H type 3 structure in human iPSCs suggest its potential as a pluripotency marker. Furthermore, rBC2LCN showed strong specificity for human iPSC-derived extracellular vesicles (EVs) but not for non-human iPSC-derived EVs. Recombinant lectins from diverse lectin families were engineered to ensure broader coverage of glycan-binding specificities. A lectin microarray with 38 recombinant lectins revealed increased expression of α2-6Sia, α1-2Fuc, and type 1 LacNac in undifferentiated human iPSCs. Moreover, increased expression levels of corresponding glycosyltransferase genes ST6Gal1, FUT1/2, and B3GalT5 were observed in human iPSCs versus that in somatic cells. 

Tateno et al. combined lectin microarray technology with flow cytometry analysis and anion-exchange chromatography and found that α2-6Sia-specific lectins showed higher binding affinity for human mesenchymal stem cells (hMSCs) with differentiation potential, suggesting that α2-6Sia α2-6 sialylation of integrin5 could function as a marker for differentiation potency of stem cells including adipose-derived hMSCs, bone marrow-derived hMSCs, and cartilage tissue-derived chondrocytes. A lectin microarray showed increased GlcNac protein modification and α-1-2-fucosylation but decreased α-1-6-fucosylation, α-2-6-sialylation, and α-1-6-mannosylation during ESC adipogenesis. 

CSCs have been reported to drive tumor initiation and growth. Lectin microarray coupled with FACS analysis revealed that sialylated glycan-recognizing lectins, MAL−I, SNA, Sambbbussieboldiana (SSA), TJA−I, Agrocybecylindracea (ACG), ABA, and Maackiaamurensis (MAH) displayed higher affinity to CSCs in CD133+CD13− Huh7 human liver cancer cells than to CSCs in CD133+ cells. Subsequent validation led to the proposal of SSA lectin as a candidate marker for CSC recognition from heterogeneous cell types.

5. Conclusions and Outlook

Glycoproteomics is an emerging field in post-genome science. Glycosylation is the most abundant and complex posttranslational modification that plays a primary role in regulating lipids, proteins, and cell functions. Lectin has been found to be very useful for detecting specific glycosyl structures. Biomarker discovery is a new exciting application of lectin microarray technology. This can potentially contribute to fundamental biological and clinical applications including the discovery of cancer diagnostic biomarkers and new drugs. We have discussed in the present review about lectin microarray as a potent tool for the discovery of glycosylation-related biomarkers. However, the current limitations of this technology must be addressed to expand its applicability.

Firstly, since most of the current natural lectins are derived from plants, it is difficult to obtain a comprehensive repertoire that is representative of glycome complexity. Thus, the repertoire of unique lectin probes on microarrays must be continuously expanded to cope with the number of glycosyl epitopes present in humans. Specifically, the discovery of novel natural lectins and their biochemical properties, cloning, and purification of all known and predicted lectin or lectin-like proteins, as well as the rational design and development of new recombinant lectins, are necessary to continue expanding the list of known and available lectins. The scope of lectin research should be extended to humans. Most of the known and predicted human lectins are ligands and receptors in cell membranes or body fluids, which participate in regulating cell functions, protein levels, and host-pathogen interactions. Thus, human lectin-based microarrays have broad application prospects in disease biomarker research.

Secondly, due to a limitation in sample quantity in many clinical situations, a normal basic lectin microarray is not sensitive enough to detect low abundance target cells or proteins. Thus, the current format of lectin microarrays must be substantially improved.

Thirdly, information on glycan structure and details of glycosylation patterns cannot be obtained from lectin microarray analysis. Thus, a lectin microarray is coupled with MS to satisfy the requirements of a fast, low-cost, accurate, and high-throughput method.

Fourthly, our goal is to elucidate highly sensitive and specific clinically relevant glycan diagnostic disease biomarkers. The sera of advanced cancer patients often contain complex non-cancer-related protein patterns. Unfortunately, the histopathological status is often overlooked during analysis of sera from advanced cancer patients. Advancement in technology facilitated the screening and elucidation of potential candidate biomarkers. However, useful disease markers account for less than 1 percent of the identified markers. Since proteins that exhibit either specific or preferential expression in cancer cells can be potential biomarkers, high-quality and high-content sample collection is a prerequisite to ensure successful glycan-based biomarker identification via lectin microarray screening.

Lectin microarrays provide a fast, high-throughput, and inexpensive tool that can support the discovery of glycol-biomarkers as well as obtain better understanding of glycans’ structure and function in various biological processes and diseases.

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Conflict of Interest

The authors declare no conflict of interest.

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