

REVIEW

Application of cancer-associated glycoforms and glycan-binding probes to an in vitro diagnostic multivariate index assay for precise diagnoses of cancer

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Personalized medicine has emerged as a widely accepted trend in medicine for the efficacious and safe treatment of various diseases. It covers every medical treatment tailored according to various properties of individuals. Cancer-associated glycosylation mirrors cancer states more precisely, and this “sweet side of cancer” is thus intended to spur the development of an advanced in vitro diagnostic system. The changes of glyco-codes are often subtle and thus not easy to trace, thereby making it difficult to discriminate changes from various compounding factors. Special glycan-binding probes, often lectins, can be paired with aglycosylated antibodies to enable quantitative and qualitative measurements of glycoforms. With the in vitro diagnosis multivariate index assay (IVDMIA) considered to be capable of yielding patient-specific results, the combinatorial use of multiple glycoproteins may be a good modality to ensure disease-specific, personalized diagnoses.

Received: December 31, 2015

Revised: March 9, 2016

Accepted: March 17, 2016

Keywords:

Aglycosylated antibody / Biomarkers / Cancer / Glycoproteomics / IVDMIA / Lectin

1 Introduction

Cells or organisms leave traces as a consequence of biological or pathological processes, including diseases. Alternatively, these traces can be a driving factor, causing such processes to occur. When specific to a certain health state, the trace, often termed as biomarker, mirrors the existence, dynamic changes, and effects of the associated process. That

is, biomarker is a measurable indicator of specific biological state, previously defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” by the National Institutes of Health Biomarkers Definitions Working Group [1]. Biomarkers can take a variety of molecular forms, including nucleotides, proteins, hormones, and metabolites. Evidence that even cells can be utilized as biomarkers has recently been found [2], since the first report that tumor-like cells were detected in the blood of a cancer patient after death [3].

Biomarkers can be used for various purposes, including disease prediction, prognosis, pharmacodynamics, diagnosis, and screening. In addition to their clinical utility, biomarkers should satisfy several criteria, such as clinical and analytical validity. Clinical validity is usually determined by performance indices including the sensitivity, specificity, positive predictive value, and negative predictive value indices. Sensitivity is a numerical index obtained from true-positive test results and represents the ability to classify an individual case as a “disease” case correctly. On the other hand, specificity is associated with true-negative test results, representing the ability correctly to classify an individual case as “non-disease” case [4]. Positive predictive value and negative predictive values refer to the probability that a positive or negative test result reflects the correct disease state [5]. A biomarker is

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Abbreviations: **AAL**, Aleuria aurantia lectin; **AFP**, alpha-fetoprotein; **A2M**, α -2-macroglobulin; **AOL**, *Aspergillus oryzae* l-fucose-specific lectin; **CRISPR**, clustered regularly-interspaced short palindromic repeats; **Dx**, in vitro diagnostics; **GalNAc**, N-acetylgalactosamine; **HCC**, hepatocellular carcinoma; **HPA**, Helix pomatia agglutinin; **HPX**, hemopexin; **IVDMIA**, in vitro diagnosis multivariate index assay; **LCA**, Lens culinaris agglutinin; **LfDB**, Lectin Frontier DataBase; **MGL**, Macrophage galactose binding lectin; **PHA**, phytohemagglutinin; **PhoSL**, Pholiota squarrosa lectin; **PNGase**, protein N-glycanase; **PSA**, prostate-specific antigen; **sLe^a**, sialylated Lewis A; **SNA**, Sambucus nigra; **sTn**, sialyl-Tn

usually approved for a specific use or for decision-making purposes only when it can clearly and correctly define who can benefit from its applications. More importantly, performance indices should make it possible for users to assess the costs of false-positive or negative results during the implementation of tests. Due to the reciprocal nature of sensitivity and specificity, it is not common for a biomarker to exhibit sufficiently high values on both indices. Sensitivity is often compromised by specificity, and vice versa [4]. For this reason, biomarkers are more commonly used for monitoring rather than for the early detection [2]. Nonetheless, the concomitant acquisition of sensitivity and specificity is an ultimate goal in the biomarker development.

Biological heterogeneity and variability are fundamental aspects of organisms, constituting the core principle of evolution and natural selection. Likewise, a disease is often developed through multiple pathways or causes. In particular, it is noteworthy that solid tumors originating from one cell show heterogeneity at the single cell level. Accordingly, the combinatorial use of molecules that cover all linked pathways can provide an overall picture of a disease. This is the underlying concept for in vitro diagnostics (Dx) composed of multiple biomarkers [6]. This perception appears as various attempts to develop multiplexed analytical techniques, including microarray [7], quantitative RT-PCR [8], and MS techniques [9]. Despite these multifarious developmental activities, we have a very short history of success with multiplexed biomarkers assays [10]. It was quite recently that we gained a clear understanding of an IVDMIA [10].

One additional noteworthy point is that a biological state can be reflected not only by changes in the biomolecular level but also by qualitative alterations. Tumor-associated glycosylation is a typical example [11], constituting a main subject in glycoproteomics which deals with identifications of aberrant glycoproteins, structural analyses of protein glycans, and implications in diseases. These tasks have been suggested to be implemented by systematic glycan profiling tools including quantitative RT-PCR, lectin microarray, and MS/MS [12]. In this review, recent achievements associated with IVDMIA are introduced, and the possibility of the use of cancer-associated glycans of glyco-biomarkers in a multiplexed manner is presented. Possible platforms or methods that can help implement glycan-based IVDMIA by the combinatorial use of suitable probes and aglycosylated antibodies are also suggested.

2 Tumor-associated glycosylation as cancer biomarkers

Dynamic alterations in protein glycosylation are a hallmark in cancer. Cumulative evidence indicates that tumor-associated glycosylation functionally contributes to cancer development, progression and metastasis [11]. In parallel, decades of research have seen vigorous efforts to exploit tumor-associated glycosylation as a cancer biomarker for various clinical applications [11]. Subtle changes in the protein glycosylation, in

themselves, do not evoke severe cellular phenotypes. Instead, they may affect extensive aspects, *albeit* minutely for each aspect, of molecular networks consisting of carbohydrate-interacting lectins and their associated proteins. Independently of these etiological issues, specific glycoforms with sufficient clinical performances have been used as cancer biomarkers. We have a typical example, the core-fucosylated glycoform of alpha-fetoprotein (AFP-L₃), whose level increases in the blood of hepatocellular carcinoma (HCC) patients [13]. Although AFP is intended for HCC patients, the serum glycoprotein can also be elevated under non-HCC conditions such as ovarian and testicular cancer [14]. Furthermore, it is frequently elevated under conditions of hepatitis, cirrhosis, or pregnancy [15]. In contrast, AFP-L₃ is more specific to HCC [16], and the AFP-L₃/AFP ratio is indicative of poor prognosis in affected patients [17]. Even patients with a low AFP level may be predicted to develop aggressive HCC if they have a high AFP-L₃ ratio [16]. Nonetheless, there remain limitations on the fuco-form with regard to its wider clinical uses, which arises from the fact that this single biomarker cannot cover all HCC patients [18, 19]. A meta-analysis indicated that there was a gain of specificity but a loss of sensitivity when AFP-L₃ is used instead of AFP [20]. However, the adoption of the micro-total analysis system (μ -TAS) contributed to an increased sensitivity in the diagnosis of HCC [21, 22], compared to the conventional liquid-phase binding assay. Thus, the use of AFP-L₃ in combination with a suitable method is considered to provide clinical utility in the diagnostic and follow-up efforts [23].

Specific glycoforms of prostate-specific antigen (PSA) have been suggested to enable more precise diagnoses of prostate cancer. Determinations of PSA levels in the peripheral blood often fail to distinguish patients with prostate cancer from those with benign prostatic hyperplasia [24]. However, α 2-3-sialylation [25], α 1-2-fucosylation, and β -N-acetylgalactosaminylation [26] of PSA are exclusively found in the sera of patients with prostate cancer. In addition, the investigation of *Sambucus nigra* (SNA)-bound PSA containing α 2-6-sialylation improved specificity in diagnoses of PSA [27], presenting the possibility that the measurement of specific glycoforms may confer increased specificity of PSA tests. However, these findings have not been clinically validated in a large-scale setting.

Besides the tumor-associated N-linked glycans as illustrated for AFP and PSA, diverse alterations in O-linked glycans have also been suggested. O-linked glycans account for 50–90% of the total mass of mucin-type glycoproteins, and structural alterations are responsible for the antigenic properties of mucin molecules [28]. The sialylated Lewis A (sLe^a) of mucin-1 is the targeted epitope of the CA19-9 assay, arising from the silencing of the relevant gene during early carcinogenesis in the gastrointestinal regions [29]. Because this antigenic test is not applicable to patients in the Lewis A-negative blood group and who are not tissue-specific, its utility is quite limited [30]. The biomarker shows the highest sensitivity and specificity for pancreatic cancer and is limited to

assessing treatment responses or predicting the recurrence of tumors after pancreatectomy [29]. The CA72-4 assay is used to assess the level of sialyl-Tn (sTn) antigen expressed in mucins. Elevated levels of the antigens are observed in a wide range of epithelial cancers [31]. However, the clinical utility of this tumor marker is best expressed in the staging and post-surgical management of gastric cancer patients [32].

A brief review of tumor-associated glycan enabled to reach several conclusions regarding the use of a biomarker in clinical settings: (i) tests with a known protein identity (i.e., AFP-L₃) mostly rely on single biomarkers. (ii) Consideration of a specific glycoform serves to enhance specificity and thus can be used to differentiate true-positive patients from false-positive ones. (iii) Nonetheless, the clinical utility of cancer-associated glycoforms has been hitherto limited due to unsatisfactory diagnostic performances. Given the merits of the tumor-associated glycan structure regarding the use as cancer biomarkers, more sophisticated applications of this molecular characteristic may provide more expanded clinical options in the treatment and management of various forms of cancer. A multivariate assay may be a potential strategy by which to achieve this goal.

3 IVDMA

Most commercially available cancer diagnostic kits use a single biomarker developed by biochemists. After strenuous efforts to gain an overall image of cancer biology, we have reached one single, undeniable conclusion: Cancer is, in nature, terribly heterogeneous, never explained by one single molecular pathway or a mechanism [33]. Several driver genes (i.e., Her2/neu, EGFR) cover only a limited subset of a cancer type, and we still do not know the complete repertoire of the driver genes involved. Given the heterogeneity and complexity of cancer, the expectations that one “super” biomarker with a certain levels of sensitivity and specificity is sufficient to make clinical decisions have been shattered. Rather, we have come to a robust belief that if we secure a complete list of biomarker independent each other, information collected from the combinatory use of multiple biomarkers will better assist in clinical decision-making [2]. This is the basis of the expectations of the IVDMA as an alternative solution to the current deadlock in cancer diagnosis and prevention.

According to the FDA’s guideline, IVDMA is defined as a test that “combines the values of multiple variables using an interpretation function to yield a single, patient-specific result that is intended for use in the diagnosis of disease or other conditions, or in the cure, mitigation, treatment or prevention of disease, and provides a result whose derivation is nontransparent and cannot be independently derived or verified by the end user” [34]. As illustrated in Fig. 1, the model of IVDMA basically relies on the derivation of a single-valued index, which is calculated from the sum of weighted values for each biomarker level. It is assumed that the biomarkers constituting the multiplexed tests are func-

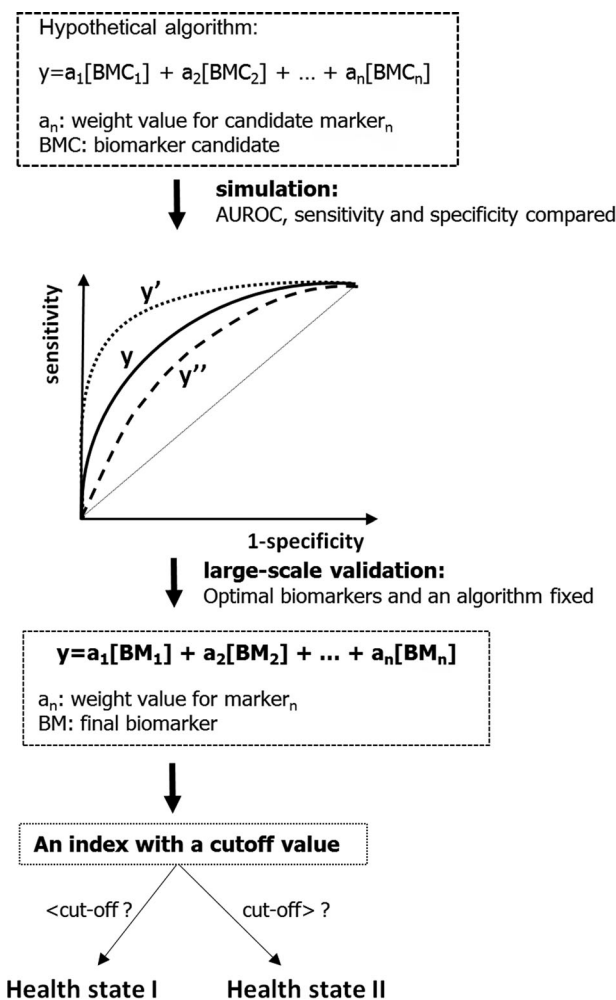


Figure 1. Scheme for an IVDMA and derivation of an index for a clinical decision-making. An algorithm to differentiate one health condition from others is drawn from a hypothetical formula, which is challenged and refined by rigorous simulations and statistical validations. An established equation provides a single index with a fixed cutoff value, which is used to differentiate or stratify individuals into two or more groups with different health conditions.

tionally independent but complementary, and that the use of such biomarkers results in better performance than a single biomarker test [35]. However, it is not easy to extract biomarkers with such properties in advance because we often do not have a full picture of a disease. Rather, the desired situation would be realized by experimental and statistic refinements. To do this, a hypothetical equation is established with candidate biomarkers that were either experimentally discovered or extracted from a known repertoire. How to derive a panel of biomarker candidates can be a matter of care especially when the candidate biomarkers are selected on a knowledge basis. They should be backed up by glyco-biological functional studies (Fig. 2). For instance, sLe^x structure has been extensively investigated in terms of involvement in hematogeneous

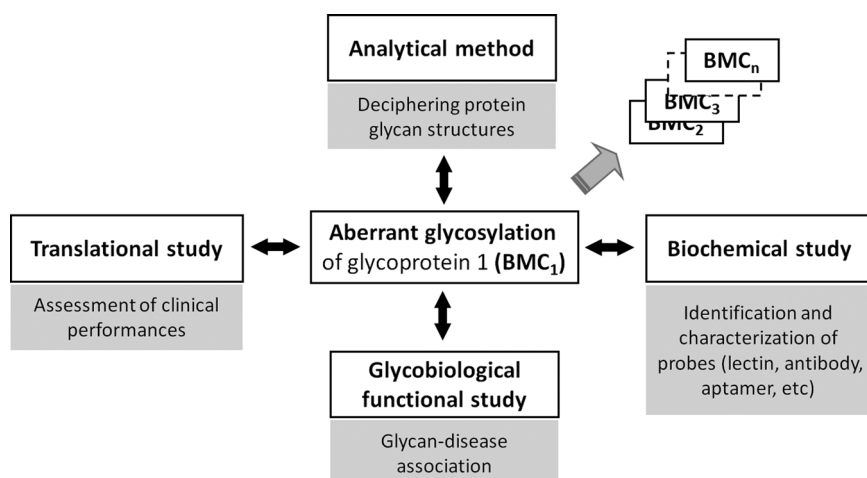


Figure 2. Knowledge-based mining of biomarker candidates for establishment of a hypothetical algorithm. A biomarker candidate can be extracted from proteins that are characterized by extensive glycobiological functional studies. The related functional studies should be accompanied by a multi-disciplinary approach comprising biochemical, analytical, and translational studies.

metastasis through interaction with selectin family members [36]. Glycobiological functional studies may well be aided by a multi-disciplinary approach accompanying biochemical, analytical, and translational studies. While constituting a candidate biomarker panel, one can set multiple algorithms from a training set. Afterward, each of the values is compared to determine the algorithm producing the most desirable clinical performances (i.e., the area under the receiver operating characteristic, sensitivity, or specificity). Because the tentative algorithm possibly contains confounding factors and biases, it should be validated and refined with validation/test sets. A final equation with a cut-off index value is derived, together with confidence and error levels, from a large-scale validation study. The cut-off value is a more reliable estimate for differentiating or stratifying individuals into different health condition groups.

There is still concern, however, that the single-valued index derived as such would not always produce better clinical performance characteristics compared to a single biomarker test [37, 38]. A large-scale study revealed that the combined use of biomarkers for ovarian cancer did not outperform the CA125 test alone [37], and there is still debate over whether the combined use of HE4 and CA125 gives better clinical performance than the CA125 test alone [38]. In addition, there exists an argument that non-disease-related artifacts may be incorporated, thereby confounding the disease status of the samples used to train the models [39]. These artifacts can originate from multiple sources beyond our estimation. Despite these concerns, a short span of time in recent years has seen “success stories” of several commercially available IVDMIAs, thereby mitigating the concern over the use of IVDMIA for cancer diagnoses. Here, a brief introduction to the IVDMIA-based cancer tests that pioneered this field will be given.

3.1 Gene-based IVDMIA: Oncotype Dx

Oncotype Dx is a type of IVDMIA intended for gene expression profiling of multiple biomarkers for breast cancer; it was not FDA-cleared but is commercially available under the

regulation of the Clinical Laboratory Improvement Amendments [40]. The Oncotype Dx test is an RT-PCR-based assay targeting 21 genes including five reference genes. A recurrence score number is derived from a mathematical formula that comprehensively evaluates the relative expression levels of 16 genes. In the formula, the 16 genes are divided into several groups, each of which is assigned different weight values based on previous training studies conducted with 447 patients [41, 42]. The score value is used to classify patients into low-risk (score <18), intermediate-risk (score 18 to 30) or high-risk (score ≥31) groups [41, 43]. A retrospective study was conducted with 668 tamoxifen-treated patients in the National Surgical Adjuvant Breast and Bowel Project B14 trial, finding that a high score was correlated with the recurrence of early-stage breast cancer and chemotherapy benefits [41]. In this regard, the Oncotype Dx assay is used to predict the recurrence of tamoxifen-treated patients who are estrogen receptor-positive and lymph node-negative [41]. A recent study has also confirmed that the test enables prediction of the risk of recurrence in women with ductal carcinoma in situ who are treated with local excision [44]. This assay was the first Dx in which multiple biomarkers were quantitatively measured with different weight values given to each biomarker to derive a single decision-making index.

3.2 Protein-based IVDMIA: the OVA1 test

OVA1 examines five ovarian cancer biomarkers: CA125, pre-albumin, Apolipoprotein A1, beta-2 microglobulin and transferrin. The measurement data obtained on an immunoassay platform are subsequently interpreted by a proprietary software using a multivariate index assay algorithm. The calculated score provides an index within the range of 0–10 [45, 46]. For premenopausal women, a score ≥5.0 is considered to denote a high probability for malignancy for premenopausal women, while a score ≥4.4 is associated with higher risk for malignancy in postmenopausal women [46]. A significant percentage of the malignancies that the CA125 test failed to identify were detected by the OVA1 test, whose

clinical performance was consistent in early- and late-stage cancers [46]. Moreover, OVA1 outperformed the CA125 test for predictions of ovarian malignancy in patients with an adnexal mass [46–48]. However, OVA1 showed lower specificity, compared to the CA125 test, instead of showing clear evidence of high sensitivity [37]. This low specificity resulted from the strategic choice of cut-off values which satisfy the need for high sensitivity during the construction of OVA1 [44]. OVA1 is intended to evaluate the risk of ovarian cancer preoperatively in patients who have a pelvic mass and are scheduled for surgery [43, 44].

3.3 Oligosaccharide-based IVDMIA: Fuco-index from triple HCC biomarkers

Vigorous attempts have been made to utilize a specific glycoform as a cancer biomarker. Most tests aimed at one type of glycoform of a single biomarker, such as AFP-L₃. However, an interesting study aiming to develop an oligosaccharide-based IVDMIA was conducted by Lee et al. [49]. In the study, a normalized fuco-form for AFP, hemopexin (HPX), and α -2-macroglobulin (A2M) was derived by the following equation.

$$\text{Normalized fuco - form} = \log_2 \left(\frac{\text{Fuc-B}_{\text{HCC}}}{\text{Fuc-B}_{\text{nor}}} \right) - \log_2 \left(\frac{[\text{B}]_{\text{HCC}}}{[\text{B}]_{\text{nor}}} \right)$$

In this equation, Fuc-B and [B] indicate the fuco-form and the total amount of the biomarker (B), respectively, in normal (nor) or HCC sera. Each fuco-form value for triple glycoproteins was variably weighted and fuco-indices were extracted from the sum of the weighted values. AUROC values for the indices were compared, and modeling using training and test sets revealed that weight values of 0.53, 0.29 and 0.18 for AFP, HPX, and A2M produced the highest AUROC value, thereby rendering the following formula:

$$\text{Index (I}_f) = 0.53 F_{\text{AFP}} + 0.29 F_{\text{HPX}} + 0.18 F_{\text{A2M}}$$

Here, F refers to the normalized fuco-form value derived from the equation above. When the cutoff value was set to 0.38, it rendered improved discriminatory power over the AFP or AFP-L₃ test alone. This pioneering study is meaningful because an appropriate combination of multiple glycoproteins can be used to develop an IVDMIA-based detection system when a specific glycoform is adequately quantified.

4 Requirements for the implementation of aberrant glycosylation-based tests for IVDMIA

As for other diagnostics, there are general requirements to be met when implementing glycan-based cancer diagnostics, including analytical factors such as analytical sensitivity, reproducibility, and precision among others as well as non-analytical factors, such as cost-effectiveness, high-throughput

handling of specimens, and additional issues related to acquisition, pre-treatment, and stability. However, there are also features unique to glycan-based cancer diagnostics. These include the selection of an IVDMIA-compatible platform and the availability of probes and aglycosylated antibodies. These requirements are perceived minimal, but critical factors to develop and implement a glycan-based cancer diagnostics system.

4.1 IVDMIA-compatible platforms

The concept of “IVDMIA-compatible” platforms is associated with multiplexed analyses of analytes. Quantifications of multiple targets should be feasible and a single index should be easily derived. Here, a specific glycoform connotes two molecular structural identities: (i) what is the protein? and (ii) which glycan structure is attached to the protein? Accordingly, to quantify a specific glycoform necessitates the identification of two different but covalently linked molecular identities, oligosaccharide and protein, and the quantification of the glycoform at the same time. Mass spectrometry (MS) could be one of the most solid technical options, and significant improvements in MALDI-TOF-MS or LC-MS/MS techniques have facilitated the identification of glycoproteins and the characterization of glycan structures with high sensitivity and specificity over the years [50]. These in-depth investigations are currently used to perform comprehensive analysis of glycoproteomic biomarker screening from sera [51]. Recently, it has become possible to identify amino acid sequences and oligosaccharides simultaneously [52]. Nonetheless, the quantification of a specific glycoform using a mass spectrometer is not straightforward due to high cost of the equipment and the difficulties associated with the acquisition and availability of standard glycopeptides or glycoproteins of a specific glycoform. Furthermore, there is a sensitivity and complexity issue [53]. Accordingly, detailed discussion will be focused on the combinatorial use of antibodies and glycan probes, such as lectins.

The simplest platform at hand would be to apply a sandwich ELISA-based immunoassay. In this case, a lectin is applied to probe a specific glycoform among a repertoire of glycoforms captured by an antibody. The antibody-lectin pair confers dual specificity toward the protein and oligosaccharide: The capture antibody confers specificity toward the bound glyco-biomarker and the lectin does this toward a targeted glycan structure. To make this approach realistic, it is essential to use an aglycosylated or deglycosylated antibody to prevent from cross-reactivity between the capture antibody and lectin probe [54]. Details pertaining to the aglycosylated antibody will be discussed in the corresponding section.

Apparently different, but similar in nature, analytical tools are available for this purpose. Various beads including magnetic or carbohydrate-based beads, can serve as an alternative solid support [55, 56]. Moreover, with the use of different beads on which different antibodies are labeled, the

simultaneous measurement of multiple biomarkers becomes possible [57]. An on-chip lectin microarray is a fascinating tool with which to analyze a variety of glyco-codes of a particular biological system [58]. However, the fact that protein identification is not always possible has limited the clinical use of this method. Among bead-based lectin arrays, there is a more sophisticated tool which uses lectins conjugated onto fluorescent dye-coated microspheres. The microspheres constitute a three-dimensional structure, making them more accessible to the target glycoproteins as compared to two-dimensional microarray surfaces [59]. These technologies also enable direct, rapid, high-sensitive glycan pattern profiling [56], and furthermore are compatible with a multiplexed assay [60]. Fluorophore-coupled lectins have been substituted for lectins labeled with metal-chelating polymers, which were measured by inductively coupled plasma MS [61]. In parallel, biotinylated lectins were used for glycan profiling [55]. These methods were intended to overcome low sensitivity of lectin and to produce high clinical performance levels.

Low analytical sensitivity has been regarded as a decisive hurdle in the routine use of MS for the analysis of glycoforms. Moreover, glycoproteins show heterogeneity; a single glycosylation site is sometimes partially occupied [62]. Thus, it becomes even more difficult to quantify a specific glycoform, which often exists at a sub-stoichiometric level even for a sophisticated mass spectrometer. These structural features make it difficult to efficiently separate, identify, and quantify the glycoprotein from bio-fluids. Many separation tools for the enrichment of glycoproteins and analytical tools for mass analysis of separated glycoproteins have been developed to overcome this bottle-neck. A lectin-immobilized capturing method has been developed to enrich a desired glycoform, which can be combined with other chemical reaction-based technologies using hydrazide and boronic acid [63]. A multi-lectin column can be employed to capture pan-glycoproteins without targeted glycoforms [64]. Instead of immobilized lectins, free lectins can be used to trap a glycoform. The separation of free lectin-glycoform conjugates was attempted by ammonium sulfate fractionation or by filtration on a membrane filter with a low molecular mass cutoff [65]. This approach is also amenable to semi-automatization on a microarray basis [66]. Finally, a targeted MRM-based method combined with lectin capturing was used to quantitatively measure the abundance of the aberrant glycoforms of target glycoproteins [67].

4.2 Glycan probes

Tumor-specific glycoforms are observed for both N-linked and O-linked glycoproteins, but the feature is displayed differently in both types. One or more glycans are additionally attached to yield heavier N-glycans [11], which can be classified into the following groups. (i) Additional branching catalyzed by N-acetylglucosaminyltransferase-3 [68] and N-acetylglucosaminyltransferase-5 [69], (ii) fucosylation by

fucosyltransferase-8 [70] and other fucosyltransferases [71], (iii) terminal sialylation with different spatial conformations [72]. Besides sialyl-Lewis^{a/x} structures [73], the incomplete synthesis of O-linked glycan chains is observed in cancer cells, yielding T, Tn, and sialyl-Tn antigens [74]. The Tn antigen is formed by the addition of N-acetylgalactosamine to serine or threonine residue, and the addition of α -2-6-linked sialic acid or β 1-3-linked galactose to the Tn antigen yields sialyl Tn (sTn) antigen or T antigen, respectively [75]. Lectin probes recognizing the aforementioned aberrant glycan structures are compiled in Table 1, which are to be discussed briefly.

Several bean plants have E- and L-type phytohemagglutinin (PHA) and the tetrameric compositions of monomeric lectins show different glycan-binding specificity [76]. L₄-PHA has widely been used specifically to recognize the β 1-6-GlcNAc linkage of N-linked glycan, and E₄-PHA has been validated to probe the bisecting GlcNAc. Fucosylation on N-linked chains are often found in several types of cancer [77]. Several lectins that can target fucosylated glycoforms have been discovered, including *Lens culinaris* agglutinin-A (LCA), *Aleuria aurantia* lectin (AAL), and *Aspergillus oryzae* l-fucose-specific lectin (AOL). Because core fucosylation is more clinically correlated with cancer than branched fucosylation, LCA has been widely used to this end [78]. However, it has been suggested that AOL has the strongest preference toward core fucosylation [79]. Additionally, it is important to note that *Pholiota squarrosa* lectin (PhoSL) binds only to core α 1-6-fucose and not to other types of fucosylated oligosaccharides, such as α 1-2-, α 1-3-, or α 1-4-fucosylated glycans [80]. Sialic acid-binding lectins are widespread in viruses, bacteria, fungi, plants, and animals [81]. However, plant-origin lectins are more specific to sialic acid linkages that are produced in human cells, including lectins from *Maackia amurensis* (MAL), *Sambucus nigra* (SNA), *Sambucus sieboldiana* (SSA), and *Sambucus canadensis* (SCA). Moreover, lectins from *Artocarpus integrifolia* (jacalin) and *Triticum vulgare* (WGA) are occasionally used despite their relative low specificity toward sialic acid [82]. Sialyl Lewis^{a/x} structures are generated on N-linked and O-linked glycans as well as glycolipids [83]. The functional involvement of the relevant structures during hematogenous metastasis was addressed in several outstanding functional studies in certain types of cancer [83]. Selectin-family members could be engineered and utilized to probe the tetra-saccharide structures. Tn, sTn, and T antigens are highly expressed in certain types of carcinomas but are not present in normal tissues or cells [84]. Lectins from *Salvia sclarea* (SSL) and *Vicia villosa* (VVL) are well-known reagents with which to probe Tn antigen [85]. The *Helix pomatia* agglutinin (HPA) was originally reported to have specificity toward T and Tn antigens [86] but was later found to have broader specificity toward diverse glycans [87]. Two different Macrophage galactose binding lectins (MGL), mMGL-1 and mMGL-2, show different specificity [88]: mMGL-1 binds Lewis^{a/x} structures, whereas mMGL-2 recognizes Tn- and T-antigens. SNA can be used to detect cancer-associated sTn-antigen in sera [89] and circulating cancer-associated sialylated glycoproteins at a very low

Table 1. Probes for cancer-associated glycan antigens and ligand specificity

Type of Glycan	Ligand specificity	Probes	Reference	
N-linked glycan	–Branching	GlcNAc β 1-4Man	E ₄ -PHA [76]	
		GlcNAc β 1-6Man	L ₄ -PHA [76]	
		GlcNAc β 1-4[Fuc α 1-6]GlcNAc	LCA [78]	
	–Fucosylation		AAL [78]	[78, 79]
			AOL [80]	[78, 79]
			PhoSL [81]	[81]
	–Sialylation	GlcNAc β 1-4[Fuc α 1-3/4]GlcNAc	AOL [81]	[81]
		Neu5Ac α 2-3Gal β 1-4GlcNAc	MAL [81]	[81]
		Neu5Ac α 2-6Gal	SNA [81]	[81]
		Neu5Ac α 2-6Gal	SSA [81]	[81]
		Neu5Ac α 2-6Gal	SCA [81]	[81]
	–Sialyl Lewis	internal GlcNAc > Neu5Ac	WGA [81]	[81]
		Gal and Man > Neu5Ac	Jacalin [81]	[81]
		Neu5Ac α 2-3Gal β 1-4[Fuc α 1-3]GlcNAc	Selectin [89]	[89]
		Neu5Ac α 2-3Gal β 1-3[Fuc α 1-4]GlcNAc	mMGL-1 [89]	[89]
		CA19-9 [92]	[92]	
		CA15-3 [92]	[92]	
O-linked glycan	–Tn		mMGL-2 [89]	
			SSL [85]	
			VVL [85]	
			HPA [86]	
	–Sialyl Tn	Neu5Ac α 2-6GalNAc-O-Ser/Thr	SNA [89]	[89]
			B72.3 [93]	[93]
	–T	Gal β 1-3GalNAc-O-Ser/Thr	mMGL2 [89]	[89]
			HPA [86]	[86]

abundance [90]. The use of galectins and siglecs is potentially possible, but little validation has been performed with regard to the in vitro diagnostics. Additionally, it is noteworthy that *Wisteria floribunda* agglutinin (WFA), obviously binding to GalNAc β 1-4GlcNAc, is used for a commercially available liver fibrosis test designed to measure WFA-positive Mac-2 binding protein [91].

Besides lectins, monoclonal antibodies (mAbs) that are either naturally occurring or specially developed have been applied to probe certain cancer-specific glycan structures. Antibodies against several tumor-associated carbohydrate antigens have been observed in human sera. These include antibodies against CA19-9 and CA 15-3 [92]. B72.3 (TAG72) with specificity to sTn was raised against human mammary carcinoma cells [93]. In addition to the currently available antibodies, recent progress in glycomics using microarray-based platforms may facilitate the discovery of yet unknown anti-glycan antibodies that are naturally occurring under tumor-associated conditions [92].

Quantitative binding analyses provide information on binding affinity and specificity of lectins toward various glycoforms, which serves to develop a lectin or an antibody to probe a clinically relevant glycoform of biomarkers. For instance, LCA have been suggested to be a preferred probe for core fucosylation to AOL and AAL [94]. More recently, preferential binding characteristic of PhoSL was presented over LCA [80]. Selectin has been reported to bind more specifically to sialomucins with sialyl Lewis^x structure, which are fre-

quently found in the blood of cancer patients [95]. Contrary to these well-defined cases, it is unfortunate that all the lectins mentioned here are not clearly defined as to their binding properties. Nonetheless, the Lectin *Frontier* DataBase (LfDB: <http://jcggdb.jp/rcmg/glycodb/LectinSearch>) is deemed to provide comprehensive glycan-lectin binding information to guide the most suitable probe toward a targeted glycoform.

4.3 Aglycosylated antibody

A lectin-antibody pair would be applied to an ELISA-based test of a specific glycoforms. However, there is a limitation to utilize the immuno-lectin assay: The most common antibody, immunoglobulin G (IgG), is a glycoprotein, having a pair of N-linked glycans in the constant region of the heavy chain [96]. For this reason, lectin binds to antibodies used to probe or capture glycoprotein markers, thereby producing a high and uncontrolled blank value. This interference makes it irrelevant to use, *per se*, the two different bio-probes.

Several attempts have been made to overcome this hurdle. First, a protein N-glycanase (PNGase)-F treatment has been used to remove N-glycans from IgG molecules [54]. This scheme is quite simple, but the problem lies in the low cleavage efficiency: The N-glycans is located inside the Fc domain and only a small fraction is digested by the enzyme. Denatured conditions enhance the cleavage activity but aggravate the integrity and binding properties of an antibody.

Endo- β -N-acetylglucosaminidase has been suggested for alternative use under non-denatured conditions [97]. The second option is to remove Fc region from IgGs using pepsin [98]. But, a significant loss of the amount and integrity could occur during the clearing step, which may hinder the routine use of this option. Lastly, chemical modifications of N-glycans using small molecules have been attempted [99], but these applications were not proven for various lectins. Each method has its own advantages and limitations, so a suitable method can be opted based on analytical properties, simplicity, cost, etc. Whichever one adopts, the analytical validity should not be impaired.

There is another option to produce aglycosylated antibodies in a more radical manner using genome-editing technologies. Since the introduction of zinc finger nuclease, the gene-specific editing of the genome was revolutionized and has been proven in a variety of organisms [100]. The gene-editing technology became easy to use with the advent of the clustered regularly-interspaced short palindromic repeats (CRISPR)/Cas9 system [101], which is described as “democratization” in the genome-editing area. There are few restrictions on the use of the CRISPR/Cas9 system to knock-out or modify a specific gene of interest in vivo as well as in vitro [101]. Hybridoma cells that are generated by fusing an antibody-producing B cells with a myeloma cell can be a target cell for this purpose. Only if one knows the subtype of IgG, the glycosylation consensus sequence in one of the exons for constant heavy chain can be corrected according to a designed donor sequence. This approach will be advantageous because an ever aglycosylated-producing system can be established by a single manipulation step. Other merits include the facts that this option can also be applied for home-made hybridoma cells and that the antibodies generated by this method are completely aglycosylated, thereby leaving no need to perform quality control steps and additional clearing steps.

5 Conclusion

Precision medicine is an emerging trend for disease treatment and prevention as it takes into account the individual variability in molecular signatures of each person. The efficient treatment and prevention of cancer absolutely relies on an early and precise diagnosis. Omics-based approaches have produced mountainous biomarker candidates, but this work is still ongoing with a low success rate. The low efficiency with regard to cancer biomarker development could be overcome with the adoption of IVDMA, as proven by the Oncotype Dx and OVA1 tests. Furthermore, tumor-associated glycosylation may serve to realize precision medicine by enhancing the diagnostic performance capabilities of biomarkers. For tumor-associated glycans to be used as cancer biomarkers, glycoform-specific probe-aglycosylated antibody pairs should be available. Then, a suitable platform should be employed that enables an analysis in a multiplexing and high-throughput manner. A rigorously validated algorithm should

be derived to give one single decision-making index, which can be used for various clinical purposes in an area with clinical unmet needs. Looking multi-dimensionally at the “sweet-side” of cancer may facilitate personalized, precision medicine [11]. Glycoform-targeted IVDMA can be understood as one of the most promising options in this context.

We would like to acknowledge the financial support from the “R&D Convergence Program” of National Research Council of Science & Technology of Republic of Korea.

The authors have declared no conflict of interest.

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