Glycoprotein Disease Markers and Single Protein-omics*

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Glycoproteins are well represented among biomarkers for inflammatory and cancer diseases. Secreted and membrane-associated glycoproteins make excellent targets for noninvasive detection. In this review, we discuss clinically applicable markers of cancer diseases and methods for their analysis. High throughput discovery continues to supply marker candidates with unusual glycan structures, altered glycoprotein abundance, or distribution of site-specific glycoforms. Improved analytical methods are needed to unlock the potential of these discoveries in validated clinical assays. A new generation of targeted quantitative assays is expected to advance the use of glycoproteins in early detection of diseases, molecular disease classification, and monitoring of therapeutic interventions. *Molecular & Cellular Proteomics 12: 10.1074/mcp.R112.026930, 836–845, 2013.

With the expansion of targeted therapeutics and individualized treatment regimens, markers for classification of diseases and treatment response are becoming increasingly important (1). Biomarkers represent surrogate measurements of correlated, perhaps difficult-to-measure, biological processes. Clinical biomarkers ideally represent one-to-one relationships with high probabilities of capturing true associations of a marker with a specific biological process. Such relationships are, however, rare in biology, which is one of the reasons for the lengthy standardized testing process required by regulatory agencies for their approval (2). Glycoproteins have high potential to serve as markers for a variety of biological processes; they represent a rich source of information because their nontemplate-based synthesis by complex enzymatic pathways reflects expression of protein-coding genes, the availability of activated monosaccharide substrates for synthesis of glycans, the activity of the enzymatic glycosylation machinery, and multiple downstream effectors (3).

In this review, we will primarily discuss O-GalNAc and N-glycoproteins as markers of cancer and associated infectious diseases. This reflects their prominence among established biomarkers associated with tissue- and disease-specific functions and the relative ease of detecting secreted and cell surface proteins (4–6). However, there are additional glycoconjugates whose assessment becomes increasingly feasible with newly developed analytical approaches (7, 8). Here, we summarize the current clinical use and future opportunities in the use of glycoproteins as disease markers. We use these examples to introduce the concept of “single protein-omics,” which we define as comprehensive characterization of all modified forms of a protein. We argue that detailed characterization of disease-specific modifications of a single protein may be a more effective biomarker discovery strategy than proteome-wide studies, which cannot capture the richness and diversity of individual protein forms.

Pathophysiology of Glycoproteins

Glycoprotein Biosynthesis and Structural Diversity of Glycans—Mucin-type O-glycosylation and N-glycosylation occur during protein passage through the endoplasmic reticulum and Golgi compartments (9, 10). Glycoproteins are secreted or localized to outer cell membranes and the inner membranes of cell compartments, including the endoplasmic reticulum, Golgi, and lysosomes. N-Linked glycosylation is initiated by oligosaccharyltransferase, which catalyzes the transfer of a dolichol-linked glycan precursor to protein asparagine residues typically within NX(S/T) (X ≠ P) sequons (11). Nascent N-linked glycans undergo trimming by glycosidases and extension of a common core (Manα1–6(Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAcβ1–Asn) by glycosyltransferases (9). In a distinct pathway, O-GalNAc (mucin-type) protein glycosylation is initiated by a group of polypeptide N-acetylgalactosaminyltransferases that catalyze the linkage of GalNAc residues to serine or threonine residues, but no specific O-GalNAc glycosylation motif has been described (10, 12). Estimates suggest that over 50% of all proteins are glycosylated, but experimental evidence for the extent of protein glycosylation is incomplete (13). O-GalNAc glycoproteins are estimated to comprise 10% of proteins in the human proteome and 50% among those going through the secretory pathway (14). Two recent papers find, in various human tissues, ~2,500 N-glycosylated proteins each, but the sets have only partial overlap (11, 15). It is likely that additional glycosylated proteins will be identified as detection methods improve.

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Glycoproteins in Disease Context—Glycosylation is organism-, tissue-, and cell type-specific (20). This is closely tied to the varied functions that diverse protein glycoforms carry out in complex organisms (16). The activities of glycosyltransferases and many additional regulatory proteins expressed in tissue-specific patterns and levels participate in glycan synthesis, using the seven monosaccharides to build a diverse set of glycan structures (16). The variety of glycan structures on human proteins is not entirely known, although some estimates of the numbers exist (17). Each glycosylation site is typically occupied by a few major and additional minor glycan forms (18, 19). In addition, the occupancy of each site, i.e., the percentage of a glycosylation site occupied by glycans, varies and can affect the quantity of glycoforms observed in a particular context. This micro-heterogeneity creates a large variety of site-specific glycoforms of potential biological interest. The search space for glycoprotein biomarkers is therefore quite large, and elucidation of the associations between site-specific glycoform variants and disease processes is one of the driving forces of current biomarker research.

Fig. 1. Glycan structures associated with cancer diseases. A, four truncated O-glycan structures; B, representative N-glycan linkages (circled); and C, four outer arm fucosylated structures that are commonly found in cancer diseases together with their sialylated forms. Tn, GalNAc1-2Gal; S-Tn, Neu5Ac2–6GalNAc1-2Gal; T, Galβ1–3GalNAcα–Ser/Thr; S-T, Neu5Acα2–3Galβ1–3GalNAcα–O-Ser/Thr.

Fucose, galactose, glucose, mannose, GlcNAc, GalNAc, and N-acetylneuraminic acid are the primary building blocks of human N- and O-GalNAc glycans. Over 200 glycosyltransferases and many additional regulatory proteins expressed in tissue-specific patterns and levels participate in glycan synthesis, using the seven monosaccharides to build a diverse set of glycan structures (16). The variety of glycan structures on human proteins is not entirely known, although some estimates of the numbers exist (17). Each glycosylation site is typically occupied by a few major and additional minor glycan forms (18, 19). In addition, the occupancy of each site, i.e., the percentage of a glycosylation site occupied by glycans, varies and can affect the quantity of glycoforms observed in a particular context. This micro-heterogeneity creates a large variety of site-specific glycoforms of potential biological interest. The search space for glycoprotein biomarkers is therefore quite large, and elucidation of the associations between site-specific glycoform variants and disease processes is one of the driving forces of current biomarker research.

Glycoproteins in Disease Context—Glycosylation is organism-, tissue-, and cell type-specific (20). This is closely tied to the varied functions that diverse protein glycoforms carry out in complex organisms (16). The activities of glycosyltransferases depend on factors, including their quantity, localization, and substrate availability (14, 16). These factors are modulated with respect to changes in the cellular environment; disturbance of this equilibrium in disease often leads to altered glycoprotein distribution. Major changes in glycoprotein distribution are associated with congenital disorders of glycosylation, and the resultant aberrantly glycosylated proteins serve as classic examples of glycomic and glycoproteomic markers of disease (21). More subtle changes in glycosylation accompany development of multifactorial diseases like inflammation and cancer (22, 23). Because tissue- and disease-specific modifications of outer membrane and secreted proteins are common, many N- and mucin-type O-glycoforms are good marker candidates and also serve as targets for imaging agents and therapeutics (24, 25). For example, glycosylation of immunoglobulin (26) and prostate-specific antigen (27), among many other proteins, represents well documented examples of disease-related alterations in site-specific protein glycoforms. Additionally, secreted glycoproteins are accessible in serum making them excellent candidates for the development of noninvasive serological assays (28).

Diseases can cause (or result from) the following: loss/gain of glycosylation sites leading to a change in their number per protein; altered glycosylation site occupancy; the type of glycans present and their distribution on particular sites; or the quantity of specific glycoproteins. Such changes can also impact downstream processes, e.g., binding of interacting proteins, which may be used to measure glycoprotein changes indirectly (29). Mutations of the NX(S/T) sequon occur frequently in disease context (30). Nonsynonymous genomic variants create or abolish NX(S/T) sites on several thousand human proteins and can be associated with cancer risk (31). Relocation of GalNAc transferases to the ER in cancer cells is associated with increased multiplicity of O-glycosylation (14), and Liu et al. (32) reported changes in site occupancy at several N-glycosylation sites in patients with ovarian cancer. We are not aware of established biomarker assays that measure changes in site multiplicity and occupancy outside of genotyping studies of cancer risk (33).

Glycoproteins derived from tumor cells and tissues carry, depending on disease context, truncated O-glycans, glycans with altered fucosylation, including Lewis antigens, N-glycans with bisecting GlcNAc, or increased β1–6GlcNAc branching (Fig. 1). Mucin-type glycoproteins are often incompletely glycosylated in cancer cells due to altered expression of T-synthase or Cosmic chaperone that stabilizes T-synthase (14, 34). The GalNAcα1-3Galβ1–3GalNAcα–O-Ser/Thr antigens and their sialylated counterparts (Fig. 1A) are detected on cell surface glycoproteins in breast and colon cancers (12, 35). The Lewis blood group epitopes, containing Fuc...
residues α1–3 or α1–4 linked to GlcNac and/or Gal (Fig. 1C), and their sialylated forms, are found on membrane glycoproteins in a number of cancer cells (36). Increased N-acetylgalactosaminyltransferase III, responsible for addition of a bisecting GlcNac residue to N-glycans, results in decreased lung colonization by tumor cells but increases metastasis in the spleen (37). Addition of β1–6GlcNac branches by N-acetylgalactosaminyltransferase V increases in cancers of the breast (38), ovary (39), and liver (40) and is associated with enhanced tumor progression and metastasis (41). These changes are shown in Fig. 1B.

Quantitative differences in glycosylation are typically analyzed by lectins or carbohydrate-recognizing antibodies. Lectins bind specific carbohydrate epitopes; for example, Phaseolus vulgaris erythrogglutinin recognizes bisected glycans, P. vulgaris leukoagglutinin recognizes complex branched glycans, and Aleuria aurantia lectin recognizes core-fucosylated glycans (3). Recent studies using lectin arrays and multiaffinity lectin chromatography coupled to liquid chromatography-tandem mass spectrometry (LC-MS/MS) advance the use of lectins in biomarker discovery (42, 43). However, lectins typically have low affinity to single carbohydrate epitopes and display varying degrees of cross-reactivity. This complicates development of standardized lectin-based assays for use in biological samples that may contain carbohydrate epitopes differing in quantity by orders of magnitude. We are not aware of clinical assays using direct lectin recognition of carbohydrates. Nonetheless, lectin-based ELISAs have been developed, and engineered lectins with increased specificity and binding affinity should enhance biomarker discovery and reach clinical utility (44, 45).

Decades of research have led to the generation of many carbohydrate-recognizing antibodies, including several in current clinical use. It is interesting to note that many “tumor-specific” epitopes are carbohydrates. A recent study showed that 41% of antibodies to a cancer cell recognized carbohydrate epitopes (46). Such studies continue to identify new glycoconjugate-recognition cancer-specific biomarker candidates and generate valuable detection reagents (47). Detailed characterization of the targets and validation of reagents for detection of specific carbohydrate structures are essential. A study of common antibodies to cancer antigen 19-9 (CA19-9)1, used in serological screening of pancreatic cancer, showed that some recognize the expected sialyl-Lewis A antigen but others cross-react with sialyl-Lewis C and N-glycolylneuraminic acid-containing epitopes (48). Table I summarizes antibodies to the important group of Lewis-type carbohydrates tested on the Consortium for Functional Glycomics arrays. The results confirm that some commonly used antibodies to the Lewis-type antigens cross-react with other structures in this assay (Table I). This is an important consideration for biomarker discovery and even more for the performance of clinical assays where the need for validation of the reagents cannot be overemphasized (34). Detailed mass spectrometric characterization of glycoprotein epitopes, development of engineered lectins, or optimization of carbohydrate-targeting immunoassays is expected to improve the quality of glycoprotein quantification (44, 49). Alternative quantitative methods complementary to immunoassays will further facilitate use of glycoconjugates in disease monitoring (50–52). In the next section we present examples of clinically validated glycan- and glycoprotein-based clinical assays, and we discuss how recent optimization of glycoform-specific measurements of these markers attempts to improve their performance, in line with the single protein-omic concept.

**Glycoproteins as Disease Markers**—Glycoproteins are heavily represented among serological markers of cancer dis-

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1 The abbreviations used are: CA19-9, cancer antigen 19-9; sialyl-Lewis A, Neu5Acα2–3Galβ1–3(Fucα1–4)GlcNAC; CA125, cancer antigen 125; CA15-3, carcinoma antigen 15–3; CA27-29, cancer antigen 27–29; AFP, α-fetoprotein; HCC, hepatocellular carcinoma; FUT8, fucosyltransferase 8; hCG, human chorionic gonadotropin; MRM, multiple reaction monitoring.
The strategy seems to be particularly effective in combination with follow-up imaging. The individual base-line level of CA125 justifies the serial serological testing and, in combination with the risk of ovarian cancer algorithm risk stratification, represents an important concept for improved biomarker screening.

CA125 is a heavily glycosylated version of MUC16, but the exact epitope was not identified when the protein was initially characterized (64). A detailed study of CA125 expressed in the OVCA-3 cell line showed that this complex glycoprotein contains O-glycans with branched type I and type II cores, some of which are decorated with the Lewis X antigen (65). The antigen also contains high mannose and complex bisecting N-glycans in agreement with studies that observed increased P. vulgaris erythroagglutinin lectin binding to serum proteins of ovarian cancer patients (66). Treatment of the CA125 antigen with peptide:N-glycosidase F (which selectively removes N-glycans) reduced antibody binding but did not conclusively define the CA125 epitope. However, the distribution of N-glycans in CA125 is similar to that of gp120 derived from lymphoblastoid cells infected with HIV-1. Nagata et al. (66) suggest a functional impact of these CA125 glycoforms on immune response, and potential therapeutic use of monoclonal antibodies to CA125 is currently being explored (67).

Carcinoma Antigen 15-3 (CA15-3) and Cancer Antigen 27-29 (CA27-29)—CA15-3 and CA27-29 are MUC1 antigens elevated in breast cancer. MUC1 is a transmembrane protein overexpressed in many cancers; the protein contains a heavily O-glycosylated variable number of tandem repeat regions (68). Post-translational modifications of the MUC1 cytoplasmic tail modulate oncogenic regulatory pathways in response to signals from the tumor microenvironment (69). The CA15-3 epitope of MUC1 is recognized by antibodies DF3 and 115D8 via a sandwich ELISA (70, 71). The DF3 antibody recognizes an 8-amino acid epitope (Asp-Thr-Arg-Pro-Ala-Pro-Gly-Ser) that may be exposed as a result of altered glycosylation in breast carcinoma, whereas the 115D8 antibody is thought to recognize a glycosylated form of the same 8-amino acid repeat (71). In cancer, shorter O-glycans often dominate, which is thought to expose regions of the peptide backbone otherwise shielded by the glycans (72).

The CA27-29 epitope recognized by the B27.29 antibody is an 8-amino acid sequence that partially overlaps with the epitope on MUC1 recognized by CA15-3 (73). Initial studies on CA27-29 indicated that it was found at lower levels in healthy patients and higher levels in patients with breast cancer, compared with CA15-3 (74). A follow-up study concluded that the CA27-29 assay does not have high enough sensitivity and specificity to detect stage I and II breast cancer, but it can be used to monitor patients treated for breast cancer (75).

CA15-3 is elevated in 65-90% of patients with stage IV breast cancer and a smaller fraction of patients with stages I-III disease; it is also elevated in some benign breast tumors,
of cancers, including germ line cancers such as choriocarcinoma and testicular cancer (85). Elevated levels of hCGβ in combination with AFP and lactate dehydrogenase are used to diagnose and stage testicular cancer, with serum levels of hCGβ < 1000 ng/ml indicating a good prognosis in testicular cancer patients (stage I) and higher levels found in stage II (1000 to 10,000 ng/ml) and stage III (> 10,000 ng/ml) (86). Most hCG and hCGβ immunoassays in clinical use are sandwich ELISA-styles (87).

hCGs contain 28–42% carbohydrate by mass with two N-glycosylation sites on the α subunit and four O-glycosylation sites and two N-glycosylation sites on the β subunit (85). The α subunit of hCG is encoded by one gene, although eight copies of the hCGβ gene exist. The amino acid sequences of hCGβ subunits present in healthy individuals are identical; other hCGβ forms are rarely expressed. Of the five described hCG forms, two act as endocrine hormones targeting the luteinizing hormone/choriogonadotropin receptor, and the remaining three are TGFβ receptor antagonists involved in autocrine signaling (85).

Hyperglycosylated hCG is detected in testicular and ovarian cancers originating from germ cells and contains larger N- and O-linked mucin-type glycans with increased branching compared with forms found in healthy individuals (88). Site-specific differences in glycosylation have been studied in urine from patients with choriocarcinoma and testicular cancer and in pregnant women. Increased triantennary N-linked oligosaccharides at Asn-30 and fucosylated glycans at Asn-13 of the β subunit were detected in patients with testicular cancer and choriocarcinoma. In testicular cancer patients, O-glycans at Ser-138 displayed higher levels of core-2 structures compared with pregnant women (88). The site-specific glycoforms described above represent promising targets for detection of testicular and other germ line cancers.

Single Protein-omics and Targeted Quantification of Site-specific Glycoforms—Historically, many tumor-specific antibodies have been shown to bind carbohydrate epitopes (89, 90). The examples of immunoassays discussed in the preceding section demonstrate that glycoprotein analytes are of substantial clinical utility for disease monitoring, but their sensitivity and specificity for the detection of early stage cancers is often lower than desired (59, 61). Improved characterization of the targets, understanding of mechanisms of action, development of improved reagents, optimization of analytical assays, consideration of appropriate clinical variables, repeated testing addressing the variable individual base line of markers, or efficient marker combinations will improve diagnostic performance.

Proteins and their co- and post-translational modifications are rich sources of information regarding the cellular and extracellular environment. Glycoproteins are complex bioopolymers with multiple forms expected to capture information relevant to specific disease conditions. Fig. 2A presents serum glycoprotein with UniProtKB/SwissProt entry Q14624 as
an example of a potential biomarker candidate. This protein contains 22% carbohydrate by weight with four N-glycosylation sites (Fig. 2B) and several potential O-glycosylation sites (Fig. 2B) (91). It is cleaved by plasma kallikrein into two fragments, which undergo additional processing into multiple peptides quantifiable in serum by LC-MS-MRM methods (92). The potential glycoforms and related proteolytic products are shown in Fig. 2C. The complexity of potential analytes that arise from this single protein is immediately evident from this figure. Examination of all its forms is a complex task that rivals the complexity of classic proteomic experiments covering entire proteomes. A 35-kDa fragment of the protein enriched by champedak galactose-binding lectin is enriched in sera of patients with different carcinomas (93), suggesting that characterization of its glycoform may indeed be relevant to examine the disease context.

It can be argued that such single protein-omics, covering all forms of a protein, have a better chance to reveal biomarkers of disease than an examination of complete proteomes, which cannot cover the richness of individual protein forms. It is inviting to speculate that incorporation of detailed structure-function understanding into the biomarker discovery process will allow selection of relevant and quantifiable glycopeptide targets. The single protein-omics approach is reminiscent of classic experiments in which proteins were isolated from biological materials for their full characterization. This approach will have limitations as any given peptide, especially in a modified form, may not be analyzable. However, the grow-
ing arsenal of innovative workflows and analytical options opens many opportunities for single protein-omic analyses.

There are multiple examples of site-specific glycoproteomic discovery workflows that identify biomarker candidates (19, 25, 39, 94). The biomarker discovery phase is typically followed by development of non-MS-based screening methods, but it is important to note that the site-specific glycoforms can be quantified by optimized LC-MS-MRM assays (Fig. 3A). These methods target glycopeptide-specific ions, including oxonium ions, peptide b- and y-ions, and intact peptide ions (Fig. 3, B–D). Optimization of mass spectrometric MRM assays for specific classes of glycopeptides will be needed, and clinical applications of such assays may require synthesis of isotopically labeled standards. Recent studies suggest that such methods are, in general, feasible; analysis of single proteins (e.g. immunoglobulins) by LC-MS-MRM methods was reported, and additional examples are likely to follow (51, 95–97). Quantifications of some of the glycoforms may be limited to the analysis of isolated proteins, and not all glycoforms will be within methodological limits of detection, but successful applications can be expected. Site-specific quantification of relevant glycoforms will complement clinical immunoassays, as immunoassays are not readily available for such targets. Verification of site-specific glycopeptide candidates by LC-MS-MRM may be a productive approach to select candidates for targeted development of immunoaffinity reagents.

In conclusion, novel discovery strategies, including analysis of autoantibodies (77, 98), arrays (42, 99), glycopeptide enrichment coupled with mass spectrometric identification (39, 43, 100), innovations in cellular glycobiochemistry (101, 102), and chemoenzymatic methods (103–105) continue to expand the pipeline of glycoproteomic biomarker candidates. Detailed characterization of the relevant site-specific glycoforms and targeted quantification of protein glycoforms by innovative immunoassays and alternative methods, including mass spectrometric quantification, are expected to enhance current clinical diagnostic options.

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REFERENCES


functional polymorphism (Asp327Asn) in the sex hormone-binding globu-}
lectin array using supramolecular hydrogel for simple detection and pattern
profiling for various glycoconjugates. J. Am. Chem. Soc. 128, 10413–10422
harvest the plasma/serum glycoproteome. Electrophoresis 33, 1746–1754
38. Romano, P. R., Mackay, A., Vong, M., DeSa, J., Lamontagne, A., Comu-
nale, M. A., Hafner, J., Block, T., Lec, R., and Mehta, A. (2011) Devel-
opment of recombinant Aleuna aurantia lectins with altered binding
domains for glycoproteomic analysis of cell surface glycosylation and
ligands for glycan-binding receptors. Methods Enzymol. 480, 165–179
array analysis of the antigen repertoire targeted by tumor-binding anti-
41. Park, J. S., Liu, Y., Glicksman, R. A., Herbert, C., Wang, D., Chai, W., and
Fei, T. (2011) The human epithelial carcinoma antigen recognized by monocular antibody AE3 is expressed on a sulfoglycolipid in addition
monoclonal antibodies against the CA19-9 antigen show variation in binding specificity with consequences for clinical interpretation. Pro-
oblems in Quant. Anal. Meth. 11, 2212–2217
throughput quantitative N-glycan analysis of glycoproteins. Methods Mol. Biol. 899, 293–313
45. Tomiyama, A., Nakagawa, H., Matsuda, K., Sato, T. A., Nakamura, Y., and
Ueda, K. (2012) Quantitative structural characterization of local N-gly-
cans in microchetroen/itivity in therapeutic antibodies by energy-resolved oxonium ion monitoring. Anal. Chem. 84, 9655–9662
46. Vanderschaeghe, D., Laroy, W., Sablon, E., Halfon, P., Van Hecke, A.,
Delanghe, J., and Callewaert, N. (2009) GlycoFibroTest is a highly performant liver fibrosis biomarker derived from DNA sequencer-based
47. Ruhaak, L. R., Zauner, G., Huhn, C., Bruggink, C., Deelder, A. M., and
Wubben, M. (2010) Glycan-labeling strategies and their use in identifica-
mination of site-specific glycan heterogeneity on glycoproteins. Nat.
Protot. 7, 1285–1298
spectrometry in the analysis of N-linked and O-linked glycans. Curr.
Opin. Struct. Biol. 19, 498–506
antigen in serum of patients with colon cancer. Cancer Res. 41, 5489–5492
51. Magnani, J. L., Nilsson, B., Brockhaus, M., Zopf, D., Steplewski, Z.,
Koprowski, H., and Ginsburg, V. (1982) A monoclonal antibody-defined antigen associated with gastrointestinal cancer is a ganglioside con-
taining sialylated lacto-N-fucopentaose II. J. Biol. Chem. 257, 14365–14369
serum CA19-9 in the diagnosis, prognosis, and management of pan-
creatic adenocarcinoma: An evidence-based appraisal. J. Gastroint. Oncol. 6, 105–119
54. Yue, T., Partya, K., Maupin, K. A., Hurley, M., Andrews, P., Kaul, K.,
55. Bast, R. C., Jr., Feeney, M., Lazarus, H., Nadler, L. M., Colvin, R. B., and


ments of MUC1 and MUC16: implications for ovarian cancer. Cancer Biol. Ther. 9, 225–236


86. Salem, M., and Gilligan, T. (2011) Serum tumor markers and their utiliza-


