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Characterization of disease-associated *N*-linked glycoproteins

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Abstract

N-linked glycoproteins play important roles in biological processes, including cell-to-cell recognition, growth, differentiation, and programmed cell death. Specific *N*-linked glycoprotein changes are associated with disease progression and identification of these *N*-linked glycoproteins has potential for use in disease diagnosis, prognosis, and prediction of treatments. In this review, we summarize common strategies for *N*-linked glycoprotein characterization and applications of these strategies to identification of glycoprotein changes associated with disease states. We also review the *N*-linked glycoproteins altered in diseases such as breast cancer, lung cancer, and prostate cancer. Although assays for these glycoproteins have potential clinical utility, research is needed to translate these glycoproteins to clinical biomarkers.

Keywords

Disease association; Glycoproteomics; *N*-linked glycoproteins

1 Introduction

Protein glycosylation is one of the most common modifications made to proteins [1]. Glycans can be attached to proteins either via an amide group (*N*-linked glycosylation) or a hydroxyl group (*O*-linked glycosylation). *N*- and *O*-linked glycosylations are distinct protein modifications; they occur through different biosynthetic pathways, and potentially have independent functions [2]. *N*-linked glycosylation plays fundamental roles in many biological processes such as cell adhesion, cell migration, and signal transduction [3]. Abnormal expression of *N*-linked glycoproteins has been observed in various diseases, and previous studies have shown that glycoprotein changes can be used as biomarkers for disease diagnosis [4, 5]. The majority of the biomarkers used in diagnosis, prognosis, and prediction are *N*-linked glycosylated proteins [6]. Examples include carbohydrate antigen CA-19–9 used for diagnosis of colon cancer [7], prostate-specific antigen (PSA) for prostate cancer [8], α-fetoprotein for liver cancer [9], and β-human chorionic gonadotropin for germ cell tumors [10]. In addition, since *N*-linked glycoproteins are most membrane-bound proteins or extracellular proteins [11], they are accessible for therapeutic purposes, such as Her2 receptor for breast cancer therapy [12].

For in-depth characterization of *N*-linked glycoproteins to identify disease-associated glycoprotein changes, glycoproteins must be efficiently separated from other cellular components before further characterization. Several methodologies have been developed to achieve this.

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2 Methodologies used to identify *N*-linked glycoproteins associated with disease

2.1 Lectin-affinity chromatography

Glycoproteins or glycopeptides can be affinity isolated with an immobilized glycan-binding protein such as lectin [13]. Certain lectins possess affinity for particular oligosaccharide moieties, and thus various lectins bind to different structures of glycans on glycoconjugates [14–16]. Various lectins have been used in glycoprotein isolation: examples include Con A for high mannose type *N*-glycans, *Lens culinaris agglutinin* for core-fucosylated *N*-glycans, *Sambucus nigra* for sialylated *N*-glycans (Table 1). Although lectins do not possess absolute specificity, subtle differences in glycoprotein profiles can be detected [17]. Advantages of this selection approach are reversible binding, multiple affinity selectors [18], and retrievability of glycans for characterization and quantification [19]. For instance, isotopic glycosidase elution, labeling on lectin-column chromatography, and iTRAQ 8-plex isobaric tags have been used to identify and quantify *N*-glycosylation sites in lung cancer sera [20].

2.2 Hydrazide chemistry

Zhang et al. reported a method for selective isolation, identification, and quantification of *N*-linked glycoproteins via hydrazide chemistry [21]. This method involves the conjugation of glycoproteins or glycopeptides to a solid support after oxidization of the carbohydrates on the glycoproteins/glycopeptides and specific release of formerly *N*-linked glycopeptides by peptide-*N*-glycosidase F [21]. This method has been applied to the identification of glycoprotein changes associated with different histological subtypes of ovarian cancer [22], aggressive prostate cancer [23], lung cancer [24], and skin cancer [25].

The hydrazide chemistry methods can be modified to analyze cell-surface glycoproteins [26,27], sialylation-specific glycopeptides [28,29], and glycopeptides containing glycans [30]. Rather than using *N*-linked glycopeptides for quantification, Chen et al. quantified nonglycosylated peptides derived from the glycoproteins immobilized on hydrazide beads and identified glycoproteins associated with hepatocellular carcinoma (HCC) [31].

2.3 Boronic acid

Affinity ligands based on boronic acid derivatives have been used to capture carbohydrates, nucleosides, glycolipids, RNA, and glycoproteins [32, 33]. The principle of boronate-affinity chromatography is that boronic acids can form covalent ester bonds with *cis*-diols under basic conditions so that glycopeptides can be separated from other molecules [34, 35]. The boronate ester bond can be reversibly hydrolyzed under neutral or acidic conditions. Suksrichavalit et al. reported synthesis of a “clickable” boronic acid ligand by introduction of a terminal acetylene group into commercially available 3-aminophenyl boronic acid [36]. Compared to other boronic acid methods, the new clickable boronic acid approach showed superior effectiveness in separating model glycoproteins (ovalbumin and RNase B) from BSA and RNase A in the presence of crude *E. coli* proteins.

2.4. Metabolic incorporation of sugar analogs for glycoprotein isolation

Metabolic oligosaccharide engineering is an emerging strategy for glycoprotein profiling. Synthetic monosaccharides containing azide [37–40], ketone [41], and thiol [42] functional groups have been metabolically incorporated onto glycoproteins in cells and living organisms, thereby arming them for covalent reaction with affinity probes. Azido monosaccharides are useful analogs due to the small size of the azide, absence in biological systems, and orthogonality to cellular functional groups [43]. Azide-labeled glycoproteins

can be detected through the reaction with phosphines using Staudinger ligation [44] or alkynes using click chemistry [45,46].

Yang et al. used metabolic oligosaccharide engineering to identify metastasis-associated cell-surface sialoglycoproteins in prostate cancer via the metabolic incorporation of AC_4ManNAz [47]. The experimental workflow of this study is illustrated in Fig. 1 [47]. First, metabolic labeling of non-metastatic and highly metastatic prostate cancer cell lines was conducted using an azide-containing mannose analog. Second, the azide-labeled glycoproteins were chemoselectively conjugated to biotinylated alkyne. Third, the biotinylated proteins were enriched by streptavidin capture. Finally, the enriched proteins were separated by 1D gel electrophoresis, digested to peptides, and identified by LC-MS/MS. Using this method, a number of glycoproteins are identified with overexpression in highly metastatic prostate cancer cell lines. Bertozzi et al. successfully profiled the cell-surface glycoproteins in a prostate cancer cell line (PC-3 cells) and primary human prostate cancer tissue treated with peracetylated *N*-azidoacetylgalactosamine [48]. Over 70 cell-surface glycoproteins were identified, and CD146 and integrin β-4 were biochemically validated in this study.

2.5 Other methods

Other methodologies have also been used to analyze glycoproteins. SEC can be used to isolate glycopeptides as glycopeptides have increased mass compared to nonglycopeptides [49]. Hydrophilic interaction LC followed by partial deglycosylation [50] and an online combination of RP/RP and porous graphitic carbon LC [51] are chromatographic methods for glycoprotein isolation. An innovative fluorescence-based multiplexed proteomics technology was also reported for identification and differential analysis of both glycosylation patterns and protein expression levels in a single experiment using gel electrophoresis and serial staining with Pro-Q Emerald 488 glycoprotein stain and SYPRO Ruby protein stain for glycosylation and protein, respectively [52].

3 Disease-associated *N*-linked glycoproteins identified by glycoproteomics

A number of *N*-linked glycoprotein changes have been identified of association with different diseases using glycoproteomic approaches (Table 2). Studies have focused on common cancers including lung cancer, HCC, skin cancer, prostate cancer, ovarian cancer, and breast cancer. The cancer-associated glycoproteins were identified by different methodologies including lectin-affinity chromatography, hydrazide chemistry, and metabolic labeling. Many of these cancer-associated glycoproteins are extracellular proteins, such as cathepsin-L, tenascin-C, and versican [53].

Interestingly, abnormal expressions of certain glycoproteins are associated with more than one type of cancer. For example, elevated alpha-1-antichymotrypsin is associated with both nonsmall cell lung carcinoma and HCC. Upregulated galectin-3-binding protein (Gal3BP or Mac-2 BP) is associated with both HCC and ovarian cancer, whereas downregulated expression of insulin-like growth factor binding protein 3 is associated with both HCC and nonsmall cell lung carcinoma. Elevated periostin levels are associated with both aggressive prostate cancer and ovarian cancer, and elevations of versican level are associated with both breast cancer [28] and ovarian cancer [54]. Most of these proteins are extracellular matrix (ECM) proteins or interact with ECM proteins. Galectin-3 binding protein is involved in tumor cell adhesion to the ECM [55] and can enhance extracellular level of protease in HT-29 cells [56]. Periostin is an important ECM protein involved in development and adhesion [57]. Periostin interacts with many other ECM proteins, such as fibronectin, collagen V, and tenascin-C [58, 59]. Epithelialmesenchymal transition (EMT), a process of morphologic transdifferentiation, is one of the critical steps of tumor metastasis [60–62].

EMT of cancer cells can enhance invasion into the surrounding desmoplastic stroma [63]. Periostin is recently reported as a member of the EMT program and periostin expression was found to correlate closest with progression variables in nonsmall cell lung cancer [63]. The opposite mechanism, mesenchymal-epithelial transition (MET), has been recently reported for the ECM protein versican in vitro [63]. Versican may play a critical role in intercellular signaling, connecting cellular reaction with the ECM and regulation of cell motility, growth, and differentiation [64]. Versican, as a putative indicator of MET, did not behave conversely to EMT proteins. Instead, versican behaved concordant to periostin in both stroma and epithelia of nonsmall cell lung cancer [63]. Although, the function and the subcellular compartment of the epithelial protein remain unclear, expression alteration of EMT-MET proteins has been documented in both desmolastic stroma and carcinoma cells [65,66].

Changes of many glycoproteins have been identified in multiple cancer types. The question is raised whether protein changes are a general biophysical effect of cancer or whether they are specific to certain cancer types. PSA is the current screening marker for prostate cancer; however, PSA is organ specific but not disease specific [67]. Development of cancer does not actually result in higher levels of PSA while the enlarged glands in men with benign prostatic hyperplasia secrete more PSA. The prostate gland leaks PSA into the bloodstream resulting in a higher blood level of PSA in men with prostatic hyperplasia and in those with cancer. Therefore, considering the size of prostate and adjusting for the value of PSA improves the accuracy of PSA as a prostate cancer biomarker [68]. Most disease-associated glycoproteins may not be organ specific like PSA, but might be cancer specific. For specific cancer diagnosis, cancer-specific glycoproteins can be combined with other markers and additional medical approaches to increase the accuracy of tests.

4 Quantification of glycosylated isoforms may improve biomarker performance

It is worthwhile to note that particular glycosylation forms of a glycoprotein are associated with particular cancers (Table 2). Advances in proteomic technologies have stimulated a great interest not only in glycoprotein identification, but also in comprehensive analysis of glycosylation of each glycoprotein. These studies have revealed that specific glycoforms of a glycoprotein may be associated with diseases. For example, fucosylated GP73 is overexpressed in HCC [69], and fucosylated haptoglobin is associated with lung cancer [70]. Differential glycosylation of complex glycans in membrane-bound and/or extracellular glycoproteins have clinical relevance [71–73]. Using antibodies against glycoproteins and glycans, Lim et al. found that serum levels of certain glycoforms of soluble CD44v increased in particular cancers [74]. They used polyclonal anti-CD44v antibody as an immobilized capture antibody and antiglycosylation antibodies as detection antibodies. Sera from patients with cancers had significantly higher levels of soluble CD44v molecules carrying cancer-associated glycotopes—sialy Lewis x and sialy Lewis a—compared to normal individuals, whereas the levels of CD44v molecules carrying nonmalignant glycotopes—sialyl 6-sulfo Lewis x and disialyl Lewis a—were higher in the sera of patients with benign diseases than those in patients with cancers.

Lectin immunosorbent assay was used to analyze the different glycosylation patterns of serum PSA and PSA from prostate cancer tissue [75,76]. In these studies, PSA was first captured with a PSA monoclonal antibody and then detected by a biotinylated lectin. Recently, our group performed simultaneous analysis of total, glycosylated, and sialylated PSA from prostate cancer and noncancerous tissues [77]. Selected reaction monitoring (SRM) was used to quantify total glycopeptides from PSA and sialylated PSA glycopeptide isolated from prostate cancer and noncancerous tissues. The abundance of glycosylated PSA and sialylated PSA was different relative to total PSA in prostate cancer and noncancerous

tissues. These data showed that analysis of glycosylated PSA may improve the cancer specificity of this biomarker. Other reports have shown that specific glycoforms are associated with diseases [70, 78] and suggest that the quantification of different glycosylated isoforms of glycoproteins may provide unique information with clinical relevance.

5 Future directions

It is challenging to identify protein markers for disease diagnosis. With implementation of glycoproteomic methods, however, great progress has been made in identification of glycoproteins associated with various diseases. To increase the accuracy of diagnosis and predication of prognosis, associations of specific glycoforms with particular diseases will need to be determined. Use of a panel of proteins and glycoforms as well as other medical approaches may be combined to enhance accuracy. To identify particular cancer-specific proteins, organ-specific proteins may be identified and monitored in diseased tissues and body fluids [79]. Compared to global proteomics, glycoproteomics provide advantages of organ specificity and a focus on a subproteome to reduce sample complexity [80]. Organ-specific glycoproteins like PSA are potentially useful in disease diagnosis and should be explored as drug targets as the limited organ access will reduce the risk of side effects.

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Abbreviations

ECM	extracellular matrix
EMT	epithelial to mesenchymal transition
HCC	hepatocellular carcinoma
MET	mesenchymal-epithelial transition
PSA	prostate-specific antigen

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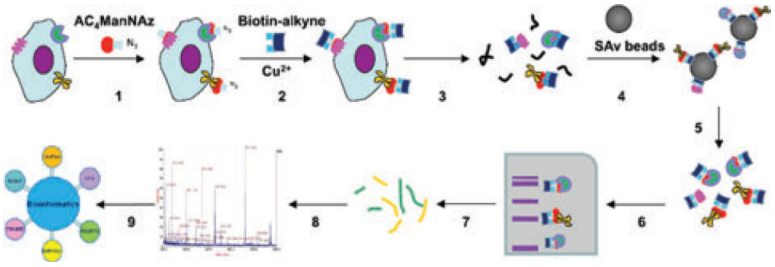

Figure 1.

Experimental workflow of analysis of cell-surface sialoglycoproteins using click chemistry. (1) Metabolic labeling of cells with peracetylated azidomannose (AC4ManNAz). (2) Chemoselective conjugation of azido sugars with a biotinylated alkyne capture reagent via Cu (I)-catalyzed click chemistry. (3) Lysis of labeled cells. (4) Affinity purification using streptavidin (SAv) resins. (5) Elution of captured sialoglycoproteins. (6) SDS-PAGE separation of sialoglycoproteins. (7) Isolation of gel slices and subsequent digestion and release of peptides. (8) Analysis of peptides by LC-MS/MS. (9) Bioinformatic analysis.

Table 1

Specificity of selected lectins used to capture specific glycoproteins

Name/abbreviation	Origin	Binding preference[a)]
LCA	Lens culinaris	Fuca1-6GlcNAc and a-Man, a-Glc
PSA	Pisum sativum	1,6-Fucosylation of the trimannosyl cor e and a-Man
AAL	Aleuria aurantia	Fuc al,6-GlcNAc4Fuc al3/l,4-GlcNAc, Fuc al,2-Gal
AAA	Anguilla anguilla	Fuc linked to the GlcNAc
LTA	Lotus tetragonolobus	Fuc a1,3/1,4-GlcNAc, Fuc a1,2-Gal, Lex
Con A	Concanavalin	Two nonsubstituted or C2-substituted a-mannopyranosyl residues in one molecule
		Man unsubstituted a t C3, C4, C6
RCA	Ricinus communis	Terminal Galb1,4GlcNAc, Gal
WGA	Wheat germ	Bisected hybrid type sugar chains, terminal a-GlcNAc or chitobiose, glycoprotein with clustered NeuAc
SNA	Sambucus nigra	NeuAc(a-2,6)Gal(b-1,4)GlcNAc4(a-2,3)NeuAc, no interaction with terminal b-Gal, b-GalNAc or NeuAc-GalNAc
MAL	Maackia amurensis	NeuAc a23Gal bl,4GlcNAc

[a)] Fuc, fucose; Glc, glucose; Man, mannose.

Table 2

Disease-associated glycoproteins identified by glycoproteomics

Protein name	Alternation	Diseases	Reference	Method used
Alpha-1-antichymotrypsin (ACT)	Upregulated	Nonsmall cell lung cancer (NSCLC)	[24]	Hydrazide chemistry
Alpha-1-antichymotrypsin (ACT)	Upregulated	Hepatocellular carcinoma (HCC)	[81]	Hydrazide chemistry
Alpha-1-antitrypsin, 40 kDa variant	Upregulated	HIV	[82]	2DE analysis
Arylsulfatase B	Upregulated	Skin cancer	[25]	Hydrazide chemistry
Cathepsin L	Upregulated	Aggressive prostate cancer	[53]	Hydrazide chemistry
CEA5	Upregulated	Mucinous ovarian carcinoma	[54]	Hydrazide chemistry
CEA6	Upregulated	Mucinous ovarian carcinoma	[54]	Hydrazide chemistry
CUB domain containing protein 1	Upregulated	Metastatasic prostate cancer	[47]	Metabolic labeling
ER-associated DNAJ (ERdj3)	Upregulated	Paclitaxel-resistant oviarian cancer cells	[83]	Fluorescence-based multiplexed proteomics and multilectin affinity chromatography
Fucosylated GP73	Upregulated	Hepatocellular carcinoma (HCC)	[69]	Lectin
Fucosylated Haptoglobin	Upregulated	Lung cancer	[70]	2DE analysis
Galectin-3-binding protein (Gal3BP) (Mac-2 BP, S90K)	Upregulated	Most ovarian cancer subtypes	[54]	Hydrazide chemistry
Galectin-3-binding protein (Gal3BP) (Mac-2 BP, S90K)	Upregulated	Hepatocellular carcinoma (HCC)	[31]	Hydrazide chemistry
Insulin-like growth factor binding protein 3 (IGFBP-3)	Downregulated	Hepatocellular carcinoma (HCC)	[31]	Hydrazide chemistry
Insulin-like growth factor binding protein 3 (IGFBP-3)	Downregulated	Nonsmall cell lung cancer (NSCLC)	[24]	Hydrazide chemistry
Mesothelin	Upregulated	High-grade serous, low-grade serous, and transitional-cell ovarian carcinoma	[54]	Hydrazide chemistry
Metalloproteinase inhibitor 1 (TIMP1), glycosylated form	Upregulated	Lung cancer	[84]	Lectin
Microfibrillar-associated protein 4	Upregulated	Aggressive prostate cancer	[53]	Hydrazide chemistry
Palmitoyl-protein thioesterase 1 (PPT1)	Upregulated	Paclitaxel-resistant ovarian cancer cells	[83]	Fluorescence-based multiplexed proteomics and multilectin affinity chromatography
Periostin	Upregulated	Aggressive prostate cancer	[53]	Hydrazide chemistry
Periostin	Upregulated	Most ovarian cancer subtypes	[54]	Hydrazide chemistry
Prohibitin 1 (PHB)	Upregulated	Liver cancer	[85]	Lectin
Prostaglandin D synthase (lipocalin-type) (L-PGDS)	Downregulated	Nonsmall cell lung cancer (NSCLC)	[24]	Hydrazide chemistry
Tenascin-C	Upregulated	Skin cancer	[25]	Hydrazide chemistry
Thrombospondin 1 (TSP-1)	Downregulated	Hepatocellular carcinoma (HCC)	[31]	Hydrazide chemistry
Triose phosphate isomerase (TPI)	Upregulated	Paclitaxel-resistant oviarian cancer cells	[83]	Fluorescence-based multiplexed proteomics and multilectin affinity chromatography
Tumor rejection anatigen (gp96)	Upregulated	Paclitaxel resistant oviarian cancer cells	[83]	Fluorescence-based multiplexed proteomics and multilectin affinity chromatography
Versican	Upregulated	Breast cancer	[28]	Hydrazide chemistry

Protein name	Alternation	Diseases	Reference	Method used
Versican	Upregulated	Most ovarian cancer subtypes	[54]	Hydrazide chemistry