Glycoengineered cell models for the characterization of cancer O-glycoproteome: an innovative strategy for biomarker discovery

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ABSTRACT

Glycosylation is one of the most abundant forms of protein posttranslational modification. O-glycosylation is a major type of protein glycosylation, comprising different types and structures expressed in several physiologic and pathologic conditions. The understanding of protein attachment site and glycan structure is of the utmost importance for the clarification of the role glycosylation plays in normal cells and in pathological conditions. Neoplastic transformation frequently shows the expression of immature truncated O-glycans. These aberrantly expressed O-glycans have been shown to induce oncogenic properties and can be detected in premalignant lesions, meaning that they are an important source of biomarkers. This article addresses the recent application of genetically engineered cancer cell models to produce simplified homogenous O-glycans allowing the characterization of cancer cells O-glycoproteomes, using advanced mass spectrometry methods and the identification of potential cancer-specific O-glycosylation sites. This article will also discuss possible applications of these biomarkers in the cancer field.

Key words: cancer biomarkers . glycobiomarkers . glycosylation

Introduction to protein glycosylation

Glycosylation is a major posttranslational modi- fication of proteins. There are two main types of protein glycosylation in eukaryotes: the N- glycosylation and the O-glycosylation. N- glycosylation consists of an oligosaccharide chain covalently linked to an asparagine residue of a polypeptide chain, occurring in the consen- sus peptide sequence: Asn-X-Ser/Thr. N-glycans share a common pentasaccharide core region and can be further processed generating three main types: oligomannose (or high-mannose), complex and hybrid [1]. These glycans are fur- ther modified, presenting different terminal structures. In contrast, O-glycosylation is char- acterized by the addition of one of six different monosaccharides: these are a-GalNAc, b-GlcNAc, a- Man, a-Fuc, b-Xyl, b-Glc and b-Gal, to a hydroxyl group of serine, threonine and tyrosine residues [2]. The different types of O-glycans are classified according to the initiating monosaccharide linked to the protein. The initiation of O-glycan via an N-acetylgalactosamine (GalNAc) is called mucin-type O-glycosylation and is distinct from the others as its initiation is controlled by an extended family of up to 20 polypeptide Gal- NAc-transferases, with potential for generating the most differentially regulated glycoproteome and lack of any clearly defined peptide consen- sus acceptor peptide sequence motif.

Mucin-type O-glycosylation (functions & specificities)

Mucin-type O-glycosylation is very diverse since it depends on the activity of enzymes codified by 50–100 distinct genes. The first step in mucin type O-glycosylation is per- formed by up to 20 polypeptide GalNAc- transferases (GalNAcTs) [3]. This highly evolu- tionary conserved family of enzymes catalyzes the transfer of N-acetylgalactosamine from the sugar donor UDP-GalNAc to the hydroxyl group of a serine or threonine [4]. This initial step is crucial for defining the site where the O- glycans are attached. The different GalNAcTs show distinct and partially overlapping peptide substrate specificities [5]. The pat- tern of expression of individual GalNAcTs shows cell and tissue specificities during development and differentiation, thus con- tributing to heterogeneity regarding sites of O-glycosylation [3]. A second level of complexity in mucin O-glycosylation is the processing

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of carbohydrate chains by other glycosyltransferases that are responsible for the definition of the final O-glycan structures (elongation and termination steps). After the first gly- can (GalNAc) is added forming the Tn antigen, eight core structures can be synthesized [1]. The most common, the core 1 structure, also known as T antigen, is synthesized by the Gal- transferase (C1GalT1), which adds Gal to GalNAc (FIGURE 1) [6].

The activity of C1GalT1 depends on a molecular chaperone COSMC [7]. Another common core structure synthesized in normal cells contains a branching GlcNAc attached to core 1 and is named core 2. Core 2 is synthesized by the core 2 b1– 6 N-acetyl glucosaminyl transferases (C2GnT). The extension of the core structures is catalyzed by b3/4 Gal-Ts and b3/4 Gn-Ts (FIGURE 1), leading to the formation of type 1 and type 2 chains. The Lewis and ABO glycan-based blood group antigens are common terminal structures, which are present in many glycoconjugates.

Biosynthesis of truncated O-glycan structures in cancer

One of the most consistent features of cancer cells is the expres- sion of immature O-glycans [8–10]. These truncated structures are known as simple mucin-type carbohydrate antigens: T antigen (Galb1-3GalNAca1-O-Ser/Thr), STn antigen (NeuAca2-6- GalNAca1-O-Ser/Thr) and Tn antigen (GalNAca1-O-Ser/Thr) (FIGURE 1) [6].

These simple mucin-type carbohydrate antigens are essen- tially not produced in normal and benign tissues, which nor- mally express more mature O-glycans. This observation explains the absence of truncated O-glycoproteins circulating in the blood of healthy individuals or individuals with benign conditions, which instead usually present more mature O-gly- cans. On the other hand, cancer cells produce, secrete and shed many different O-glycoproteins with truncated O-glycans, which reach the circulation and may be detectable in the serum. However, it is known that nonsialylated glycoproteins are cleared from circulation through receptors expressed by the innate immune system [11]. Previous studies have shown that sialylated structures ST and STn antigens are readily detectable in cancer patients' sera [12–15]. Two classical serological bio- marker assays used in cancer, CA19–9 [16] and CA72.4 [17–19], are based on the detection of sialylated O-glycans, and espe- cially the latter that detects STn demonstrates that proteins expressing the STn glycoforms circulate in cancer patients' serum.

Several mechanisms have been shown to underlie the expres- sion of truncated STn antigens in cancer, being associated with poor prognosis and tumor aggressiveness. One mechanism is the altered expression of the sialyltransferase ST6GalNAc-I, which is the main STn synthase [20,21]. Overexpression of this enzyme in cell lines can override the normal O-glycan elonga- tion machinery resulting in high STn expression [21,22]. Another mechanism may be the reduction of core 1 elongation that leads to accumulation of Tn, which serves as a substrate for ST6GalNAc-I. The core 1 synthase C1GALT1 is dependent on a specific chaperone Cosmc, and previous studies have reported hypermethylation or somatic mutations of the COSMC chaperone gene [7,23–26], leads to the expression of Tn and STn. Finally, cancer-associated relocation of the polypep- tide GalNAc-Ts within the Golgi apparatus and from Golgi to endoplasmatic reticulum may also explain the increased expres- sion of truncated O-glycans [27,28].

Engineered cell models for characterization of O-glycoproteome using advanced mass spectrometry technology

The definition of the O-glycoproteome has been a major chal- lenge in the field due to technical difficulties related to the complexity and heterogeneity of glycan functions and structures at individual glycosylation sites. The recent use of genomic editing tools has allowed the development of isogenic cell sys- tems that along with extensive application of mass spectrometry methods uses

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electron transfer dissociation (ETD) fragmenta- tion. Glycopeptide O-glycosite identification based on ETD-MS2 has been applied for high throughput site-specific O-GalNAc proteomics. These technologies have enabled the pre- cise determination of protein O-glycosylation sites in cells [29–31]. These tools have greatly evolved in the past years and show unlimited potential to revolutionize the glycobiology field.

The approach using zinc-finger nucleases targeting the knockout of COSMC gene has been applied in a number of human cancer cell lines originating from different organs [30]. These so-called isogenic cell models have been shown to pro- duce stable cells expressing homogenous truncated O- glycosylation with Tn and/or STn O-glycans [29,30,32].

These cell models have allowed the generation of unlimited amounts of material for straightforward isolation and identifica- tion of GalNAc-O-glycopeptides from total cell lysates, or even from secretions using lectin chromatography before and after sialydase treatment followed by nanoflow liquid chromatogra- phy tandem mass spectrometry with ETD for glycan site determination [33]. Alternative methodologies to lectin enrichment such as metabolic labeling with azide modified sugar (GalNAz) combined with click chemistry have been previously used for characterization of O-GalNAc glycoproteomes [34,35]. This pro- cess has been shown to be very useful for several studies. It involves a step in which the azido sugars are fed to cells or organisms for later identification of the glycoproteins contain- ing the azido sugars. This methodology has also some disadvan- tages since the cell's epimerase is able to epimerize GalNAz to GlcNAz and therefore the produced O-glycoproteome data must be analyzed with care. The enrichment using lectin chromatography has shown to have advantages over this and other methods, such as oxidation of glycans and methods based on charge affinity, as previously discussed elsewhere [36]. Using lectin chromatography followed by advance Mass Spectrometry, hundreds of unique O-glycoproteins and O-glycosylation sites have been identified in several cell line models from different tissues [30,31]. Further extensions of this strategy have been developed combining the differential analysis of the function of specific GalNAc-T isoforms, the generation of isogenic cell models with and without a specific GalNAc- T, allowing the identification of nonredundant functions of individual members of the GalNAc- T family [32,37]. Furthermore, similar strategies have been applied targeting the O-mannose gly- coproteome, revealing important targets in this additional type of O-glycosylation [30,38].

Broad knowledge of O-glycosites allows for the analysis of novel biological functions of glycosylation as the recent discovered mechanism for regulation for proprotein convertase process of proteins [37]. Furthermore, these new tools enable a fast and simple understanding of which O-glycoproteins can be found in a particular cell model and which enzymes may play a role on that specific glycosylation. This is particularly important given the complexity of O-glycosylation and the many and very efficient GalNAc-Ts controlling O-glycans sites on pro- teins [3], already addressed in this review. The O-glycoproteins found in the culture media of these engineered cell models also open for an illustration of which O-glycoproteins are secreted/shed and that have potential to be used as a biomarker. A similar lectin enrichment- based O-glycoproteomic strategy has therefore also been applied to cancer patient serum sam- ples [39]. In fact, although the cell line models allow for unlimited sample availability and extensive O-glycoproteome analyses, from a bio- marker perspective, the use of data from these model systems needs to be combined with data obtained from in vivo settings, such as cancer patients serum and tumor tissues [36].

Application of O-glycoproteins as biomarkers in cancer

Several proteomic studies used for the discovery of cancer biomarkers in the serum have been designed to interrogate the general proteome without taking into account the aberrant glycosy-

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lated forms produced by cancer cells [40]. Many cancer biomarkers currently used in the clinics are based on circulating O-glycoproteins that are detected in estab- lished serological assays (CA125, CA15–3, CEA, CA19.9) [41]. Most of these serological assays are used for cancer patient follow-up and monitoring patient response to treatment. How- ever, these serological assays may also show elevated levels in non-neoplastic and inflammatory conditions therefore limiting their use in screening applications for cancer diagnosis [41,42]. The characterization of cancer O-glycoproteomes using the engi- neered cell models described above provides a massive amount of information on potentially functionally important glycosites and on O-glycoproteins that can be used as possible targets for the development of more specific serological assays for applica- tion in cancer. The focus on specific isolated protein targets and their changes in O-glycan structures should allow for more detailed studies on cancer-specific O-glycoforms and the evalua- tion of their potential as biomarkers. This knowledge may also provide the dissection of the role of specific O-glycans conferring oncogenic properties to cancer cells and the interactions of O-glycans interfering with the immune response in patients with cancer [43].

Expert commentary

The application of genetic engineered approaches targeting spe- cific glycosylation-related genes for the generation of cancer cell models displaying homogeneous glycans has allowed the char-acterization of the cancer cell O-glycoproteome when combined with advanced mass spectrometry methods. These approaches and the information generated have allowed a major progress toward the identification of possible biomarkers in several dis- eases, including cancer.

The identification of tumor-derived glycoproteins constitutes possible biomarkers with potential-enhanced specificity and that can be detected in the serum of patients with cancer. Combination of these data with glycoproteomics analysis of serum and tissue samples, as well as complementary methods that can be applied in general diagnostic laboratories, such as those based on immunoenzymatic methods, proximity ligation assays and others, is likely to be developed targeting some of the recently identified biomarkers.

Such biomarkers may improve and enlarge the serological application targets for the detection of cancer, eventually improving early diagnosis.

Five-year view

Cell models with targeted genetic manipulation of glycosylation-related genes combined with advanced mass spec- trometry methods have opened a new era in the glycobiology field:

. The recent works applying these approaches to characterize specific function of a single glycosyltransferase showed that these strategies are going to provide important biological information regarding the function of glycosylation in physi- ological and pathological conditions;

. Further developments of the analytical equipment and bioin- formatic tools analyzing polyomic (genomics, transcriptomics, proteomics, glycomics and metabolomics) data will provide further relevant information in the context of complex dis- eases systems;

. The adaptation of the glycoproteomic analysis to serum sam- ples from cancer and normal control individuals will allow the validation of some of the recently described O-glycosites on glycoproteins identified in the cell model systems.

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Key issues

. Mucin-type O-glycosylation is a common and diverse form of posttranslational protein modification. These different structures are expressed with cell and tissue specificity and show major alterations in pathologic conditions. These major changes include the expres- sion of immature truncated O-glycans, such as Tn and STn antigens.

. The development of genetically engineered cell models, combined with liquid chromatography tandem mass spectrometry with electron transfer dissociation for glycan site specification, has allowed the characterization of the O-glycoproteome of cancer cells.

. The O-glycoproteome of cancer cells constitutes a major source of O-glycoproteins identification and opens for their potential as biomarkers of this disease and can provide massive information contained in the glyco-code of a cancer cell.

Financial & competing interests disclosure

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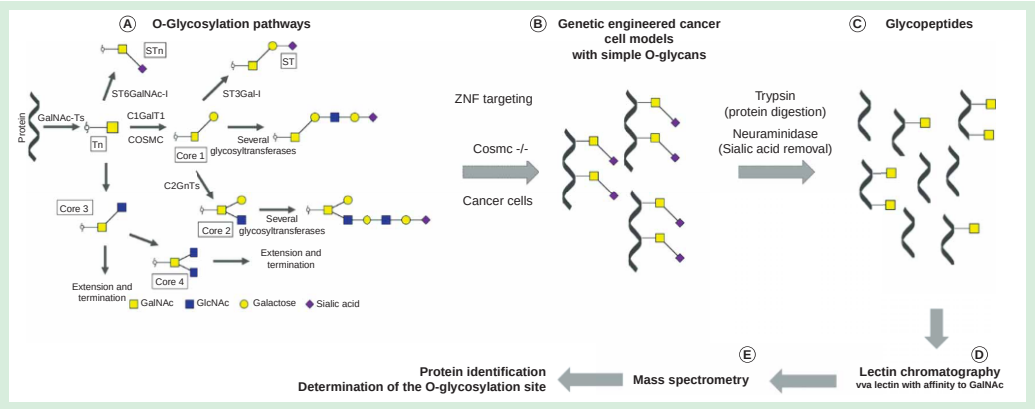

Figure 1. Cell lines O-glycoproteome characterization. Schematic representation depicting the initial biosynthetic pathways of O-linked protein glycosylation (A) and glycoengin- eering of cells used to define the O-glycoproteome of cancer cells. The genetic targeting of COSMC, a molecular chaperone of the Core1 Synthase (C1GalT1) results in a simplification of the cell O-glycosylation leading to an homogeneous truncated GalNAc (Tn) and NeuAc-GalNAc (STn) O-glycans (B). Tryptic digestion and neuraminidase treatment of the O-glycopro- teins (C) allows for further isolation of GalNAc glycopeptides using VVA lectin chromatography (D). Peptides and O-glycan sites identification is achieved by nLC-MS/MS analysis (E).

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