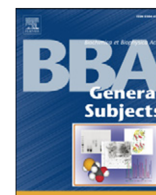
Biochimica et Biophysica Acta 1860 (2016) 1676–1687

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen



Methods for the absolute quantification of N-glycan biomarkers☆

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ARTICLE INFO

Article history:
Received 22 December 2015
Received in revised form 1 March 2016
Accepted 2 March 2016
Available online 4 March 2016

Keywords:
Glycan standards
Cancer
Mass spectrometry
Personalized medicine
Isotopic dilution

ABSTRACT

Background: Many treatment options especially for cancer show a low efficacy for the majority of patients demanding improved biomarker panels for patient stratification. Changes in glycosylation are a hallmark of many cancers and inflammatory diseases and show great potential as clinical disease markers. The large inter-subject variability in glycosylation due to hereditary and environmental factors can complicate rapid transfer of glycan markers into the clinical practice but also presents an opportunity for personalized medicine.
Scope of review: This review discusses opportunities of glycan biomarkers in personalized medicine and reviews the methodology for N-glycan analysis with a specific focus on methods for absolute quantification.
Major conclusions: The entry into the clinical practice of glycan markers is delayed in large part due to a lack of adequate methodology for the precise and robust quantification of protein glycosylation. Only absolute glycan quantification can provide a complete picture of the disease related changes and will provide the method robustness required by clinical applications.
General significance: Glycan biomarkers have a huge potential as disease markers for personalized medicine. The use of stable isotope labeled glycans as internal standards and heavy-isotope labeling methods will provide the necessary method precision and robustness acceptable for clinical use. This article is part of a Special Issue entitled "Glycans in personalized medicine" Guest Editor: Professor Gordan Lauc.
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1. Introduction

1.1. Opportunities for glycan markers in personalized medicine

Contrary to common belief, most prescription drugs and treatments show rather moderate efficacies that lie between 30 and 70% implying that large numbers of patients are not benefiting as they should from current treatment options [1]. This is particularly the case for chemotherapy and antidepressants where the efficacy can fall below 25% [2]. To increase the number of positive responders and to reduce adverse side effects to current treatment options the concept of personalized medicine had been introduced more than a decade ago. A personalized medicine approach to treatment takes into account the individual genetic and physiological makeup of each patient in the choice of treatment options. It does not refer necessarily to the development of new drugs but helps to identify those patients for treatment that are likely to benefit, from those which are unlikely to respond or where the side effects would outweigh the benefits. This patient stratification process requires linking of certain molecular traits or signatures of individual patients to the efficacy of a particular treatment. Unlike many biomarkers for diagnosis or disease monitoring, companion biomarkers should be backed by a clearly identified mechanism of action [3]. Although the development of personalized medicine has been dominated by genetic markers partly due to historic reasons and due to maturity of genomics over other omics, the limitations of a genomics-only approach to describe individual variability is becoming more and more evident [3]. In particular, as single gene mutations have been found to be insufficient for an unambiguous patient classification into responders and non-responders other additional markers are needed that reflect the non-hereditary individual variability for gender, age, weight, environmental factors, dietary habits etc. Here gene products and derivatives like RNA, proteins, and also glycans, lipids and metabolites can draw a picture that reflects far more accurately the physiological situation of an individual at a given time and adding important information to the genomic signature for patient stratification.

In this feature article we concentrate on glycans as potential companion and selective disease markers and the methods for their quantitative analysis. A particular emphasis is given to methods for the absolute quantification of glycans after release from the proteins and their potential to enter clinical practice.

Although some glycans like milk oligosaccharides or hyaluronic acid are present in free form, most are conjugated to a lipid anchor or a protein often resulting in a clustered presentation of glycan motifs that strengthens the otherwise weak mono-valent interaction between carbohydrates and their protein receptors [4]. The interaction of cell surface glycans with complementary glycan binding proteins (lectins)

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http://dx.doi.org/10.1016/j.bbagen.2016.03.003
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located on neighboring cells, other cell types, or pathogens like virus, bacteria or parasites mediates is crucial in biologically and biomedically important processes like cell–cell adhesion, cell migration, development, pathogen recognition and infection [5]. Their implication in nearly every pathological condition, consequently suggests an increasing role for glycans as disease markers [5–7]. The majority of mammalian proteins are N-glycosylated, a common post-translational modification of the asparagine residue within the consensus sequence Asn-X-Ser/Thr. N-glycans are large complex and branched glycans that share a common pentasaccharide core and present considerable micro-heterogeneity due to variations in the number of antennae, terminal glycan residues and core modifications. O-glycans are attached to threonine or serine residues, show a larger variety of conserved core structures and are often shorter than N-glycans. Glycosphingolipids (GSLs) which are composed of a ceramide moiety and a hydrophilic carbohydrate head group are found almost exclusively embedded into the lipid bilayer of the extracellular membrane [8]. GSLs are particularly abundant in the vertebrate brain where they make up to 80% of all glycoconjugates [9]. Glycosaminoglycans (GAGs) finally, are highly sulfated linear polysaccharides which are either conjugated to a protein backbone (proteoglycans) or are expressed as free reducing oligosaccharides like heparin and hyaluronic acid. Present in the extracellular matrix, GAGs are important mediators of protein–protein interactions and essential for creating defined protein gradients. These four major classes of mammalian glycans which comprise the human glycome are however so different in size, charge, occurrence and complexity that although desirable, currently no single method is available for their (simultaneous) analysis [6].

For biomarker discovery protein N-glycosylation can be analyzed on several levels of detail: on a proteomics level, that focuses on the identification of glycoproteins and their corresponding glycosylation sites, a glycoproteomics level, which determines the glycan linkage to the protein, site occupation and oligosaccharide composition on each site and finally a glycomics level which focuses entirely on glycan structures and monosaccharide connectivity, but where information regarding the glycosylation site has been lost. These techniques are complementary for studying protein glycosylation on a system level but even their sequential application will not provide a full picture of protein glycosylation that includes the unambiguous characterization of all glycan structures on all glycoproteins in a biofluid. Due to the exponential growth in complexity when moving from the proteome to the glycoproteome [10] current technologies still require a choice of focus either on the proteome or glycome. Full identification of protein and a full structural analysis of the attached glycans is currently routinely only carried out for single glycoproteins e.g. in the analysis of recombinant therapeutic glycoproteins or glycoprotein disease markers (e.g. prostate specific antigen (PSA)) [11].

Although previous glycan biomarker research has also included analysis of glycosphingolipid and glycosaminoglycan levels by mass spectrometry (MS) [12], immunostaining [13] or lectin arrays [14] the focus of most studies has been on the plasma N-glycome followed by the analysis of O-glycans on mucins which are highly over-expressed in carcinomas [15]. This preference can be explained by the high abundance of N-glycans in serum and other body fluids, a manageable number of structures and more mature techniques for sample preparation and analysis of N-glycans compared to those available for other glycan classes [6]. Human N-glycans, that are present on the majority of secreted proteins are easily obtained from a larger number of body fluids including serum/plasma, urine, saliva, tears [16], milk [17], semen or amniotic fluid [18] by enzymatic or chemical removal.

1.2. Serum N-glycans

Although estimates for the human N-glycan repertoire go as far as 2000 different structures [19], the currently experimentally accessible human N-glycome is far smaller and very dependent on the employed analytical method. The plasma N-glycome has been reported to contain more than 100 different glycan structures [20] but depending on the analytical method used only a fraction is routinely quantified due to either intense peak overlap during the chromatographic separation or a lack of sensitivity for detecting less abundant species by direct injection methods or MALDI-TOF (matrix assisted laser desorption/ionization coupled to time-of-flight detection) analysis [21]. By nano-liquid chromatography coupled to tandem mass spectrometry (nano-LC MS/MS) recently over 170 distinct N-glycan structures where registered in the plasma glycome and partially assigned by exoglycosidase digestion and diagnostic fragment ions [22]. The 20 most abundant plasma N-glycans that account for over half of the plasma glycome are mono and bis-sialylated or neutral core-fucosylated bi-antennary structures. These are major structures present on the most abundant plasma glycoproteins like the immunoglobulin isotypes IgG, IgM and IgA, transferrin, alpha-2-macroglobin, C3-complement or haptoglobin [23].

Alpha-2-macroglobin, C3-complement and haptoglobin are acute phase proteins (APPs) which are part of the innate immune system and that show expression levels that are sensitive to inflammatory processes. The high basal concentration of some APPs in plasma together with their significant changes in expression levels during inflammation ranging from 50% of ceruloplasmin to over 1000-fold for the C-reactive protein, can have an impact even on the total serum glycan levels which is measurable as an increase of mobile hexosamine N-acetyl methyl groups by nuclear magnetic resonance (NMR) [24].

1.3. Total serum vs. individual glycan analysis

Changes in the total serum glycan profile could therefore merely reflect an increase in the expression of certain high abundance glycoproteins e.g. APPs as a result of a general inflammatory process including infection, trauma, surgery, burns etc. rather than an indication of aberrant glycosylation, a hallmark of many cancers. Although the over-expression of glycosyltransferases (GT) that add sialic acid and fucose residues or lead to the expression of higher branched structures in tumor tissue can affect all proteins alike and therefore potentially lead to an amplification of the glycan marker, glycosylation is also protein specific and changes in GT expression levels will have a differential impact on the glycosylation status of individual proteins, which is largely determined by structural factors. A clear example for this behavior is the very distinct glycosylation pattern of IgG heavy chains presenting exclusively bi-antennary structures with a far lower degree of sialylation than those found on other serum glycoproteins. These protein-specific differences, that are potentially valuable disease markers, can face the chance of being diluted in a total serum analysis and are therefore best profiled separately and after immunoseparation or isolation by gel electrophoresis. Two examples for a protein-specific glycan profiling to enhance biomarker selectivity are the analysis of anti-citrulline autoantibodies in rheumatoid arthritis [25] or the glycoprofiling of PSA in prostate cancer [11]. The sensitivity of glycan analysis methods other than immunosorbent assays for the quantification of low abundance biomarkers like PSA where diagnostically relevant concentrations is in the low nanomolar range however remains a major challenge [26]. The glycan analysis of single acute phase proteins is more straightforward, e.g. haptoglobin has been found to present a significant increase in sialyl Lewis X terminating, core-fucosylated and higher branched glycans in samples from lung, prostate, hepatic, breast, ovarian, pancreatic or colon cancer patients [27].

The serum proteome is composed of hundreds of proteins with a dynamic range of concentration covering 10 orders of magnitude but dominated by a handful of proteins only. Only 22 proteins account for over 99% of protein content in plasma and albumins alone are responsible make up for over 50% of serum protein content [23]. The isolation of individual proteins from plasma in sufficient quantities and the subsequent glycan profiling is an analytical challenge and in the case of many low abundant glycoproteins exceeds the current limit of quantification of

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