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Proteoglycomics and Disease Marker: Promises and Future Challenges

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Abstract

Proteoglycomics is a systematic study of structure, expression, and function of proteoglycans, post-translationally modified subset of proteome. The process of glycan synthesis relies on number of highly competitive process involving glycosyl tranferases. Therefore, the process of glycosylation is highly sensitive to biochemical environment and has been implicated in many diseases including cancer. Recently, the interest in profiling glycome has increased for the potential of glycan as disease marker. The established technologies of proteomics and glycomics, proteoglycomics research requires unique approaches for elucidating structure-function relationship of proteoglycan components, glycosaminoglycans chain and core protein. This review discuss on the existing newly developed technologies widely involved in profiling glycome and role of proteoglycans that can be used as potential marker for disease condition.

Keywords: Proteoglycomics; Glycosylation; Glycoprotein; MALDI; Mass spectrometry

Introduction

Glycomics is comparative study of all glycans expressed in biological system. Unlike genomics and proteomics, it is technically difficult to obtain the complete profile of glycome as glycan are bound to proteins directly correlated to genome. Glycan are produced from proteins like metabolic products. These acts as unique molecules which link the three major areas of genomics, proteomics and metabolomics. Glycosylation is one of the most common Post Translation Modifications (PTMs) of secreted protein [1]. The attachment and processing of a diversity of glycans at each glycosylation site on glycoproteins leads to its modification and each glycoprotein is therefore the collection of 'glycoforms'. The glycoforms populations of glycoprotein is cell and protein specific and highly reproducible in a given physiological state, but alter in case of disease condition [2]. In various diseases, including cancer, glycosylation has been crucial event affected by biochemical environment; therefore, interest in profiling the glycome has increased owing to the potential of glycans for disease marker. In last several years major attention have been paid in biomarker identification for prognosis of disease, by using serum protein or peptide profile and mass spectrometry techniques [3]. Although, identification of marker based on mass spectrometry peaks produced by Surface-Enhanced Lased Desorption/Ionization (SELDI) or Matrix Assisted Laser Desorption/Ionization (MALDI), in case of proteins/peptide biomarkers lacks sensitivity and reproducibility which remains big issues in making this technique reliable [4]. The promise of glycoproteomics lies in identification of disease marker, as oligosaccharide can be more easily identified and quantified, by focusing on glycosylated proteins; the number of potential oligosaccharide marker to be investigated is significantly smaller as compared to the number of potential peptide or protein biomarker [6]. In this review we have tried to emphasize on the newly developed technologies for identification of potential glycobiomarker for diseases and the future challenges for development of reliable and promising biomarkers for disease [7].

Proteoglycans the Major Player in Disease

Proteoglycans (PGs) are ubiquitously found on virtually all cell surfaces and basement membrane of tissues. The interaction of PGs with proteins, mediates many biological processes including cell-cell and cell matrix interactions, growth factor sequestration, chemokine and cytokine activation, microbial recognition, tissue morphogenesis during embryonic development, and cell migration and proliferation [8-12]. To study the role of PGs in disease is challenging because of tremendous structural diversity of glycoconjugates constituted by core proteins posttranslational modified with liner, anionic polysaccharide, Glycosaminoglycans (GAGs) consisting of repeating disaccharides. The glycomics and proteomics based study of GAG chain and protein core on proteoglycans, can serve to identify new proteoglycans, catalog subset of proteome that show the level, type, and structure of GAG substitution, quantify PGs, investigate structure-function relationships, and study the impact of PGs in development or disease [13]. The post translational modification of PGs effects normal physiological condition for example, modification in the versican and decorin core proteins are linked to adenocarcinoma eucharis [14]. The integrin distributions have been affected by the posttranslational modification of lumican core protein interacting with melanoma cells [15]. Glypican is S-nitrosylated on its protein core to allow processing of polyamine uptake [16]. The promise of proteoglycomics underlies in developing new diagnostic values for diseases, recent work by Wang et al. has demonstrated the role of heparin sulfate proteoglycan (HSPG), glypican-3 (GPC3) as marker of Hepatocellular Carcinoma (HCC) [17]. Immunofluorescence microscopy imaging allow the spatial distribution and visualization of PGs in cells and tissues, currently a number of well characterized Monoclonal Antibodies (mAbs) are available commercially that react specifically with PG-related epitopes. Two IgM type mAbs,

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10E4 and 3G10 have been used in HSPG immune-detection [18-21]. Antibodies against epitopes specific to other GAG types have been generated, characterized, and used for immune-fluorescent detection of PGs in rat brain [22]. The pioneering work of has used anti-HS, domain-specific mAbs generated through phage display and bio-panning technology that has higher affinity and specificity than IgM type mAbs generated by immunization used in spatial profiling of HS structural domain in kidney [23]. Antibodies against epitopes specific to GAG types have been generated through immune-fluorescent detection of PGs in rat brain [22]. Lauer and coworker used fluorophore-assisted carbohydrate electrophoresis to examine changes in kidney HS disaccharide composition at different time point from the onset of streptozotocin-induced diabetes in rats [24].

Methods for Identification of Glycan Disease Marker

The mass spectrometry technology has large impact on the discovery and quality of glycan disease markers. Apart from proper study design, sample selection and chemical processing of samples for mass spectrometry analysis play major role in mass spectrometry analysis for discovery of glycan. Glycans are generally isolated from glycoprotein by first cleaving off the glycans and than purifying them with solid phase extraction or High Performance Liquid Chromatography (HPLC). The concentrations of samples are performed with vacuum centrifuges or lyophilizers before mass spectrometry analysis. Glycan derivatization (e.g. methylation, permethylation) to improve ionization and stability of ions in the mass spectrometer. Glycans are attached on glycoproteins via nitrogen on an asparagine N-linked glycans (N-glycans), while O-linked glycan (O-glycan) attach via oxygen on a serine or threonine, the release of glycan from glycoproteins is done by either reductive beta elimination, most commonly used for releasing O-glycan, while peptide N-glycosidase F (PNGase F) enzymatic cleavage is the most common for releasing N-glycans [4,25-27]. Ammonia- based beta elimination reaction is alternatively used against beta elimination reaction to overcome peeling reaction, where strong base cleaves off a monosaccharide from glycan producing degraded structures [28].Alternatively, hydrazinolysis can be used to release N-linked and O-linked glycans simultaneously but is rarely used owing to the relative difficulty and hazards of the process (Merry et. al., 2002) [29]. Hydrazine monohydrate substitutions have been used to decrease explosion danger of anhydrous hydrazine but were not as effective in the release of the glycans [30]. The glycoprotein samples for mass spectrometry analysis contain high level of salt, which decreases the ionization of the desired analyte. Desalting is often accomplished by filtration and dialysis, cation exchange resin or carbon- based column adsorption [4, 26-28,31,32].

Isolation of glycans from peptide or protein present in the solution is necessary because the ionization of protein or peptide occurs more readily which suppress glycan signals. The difference in polarity between peptide and glycans, has been used for purification of glycans. Large amount of protein can be precipitated from the solution using methanol, ethanol, or acetone [4,26,31,32]. Lectin affinity enrichment has also been used to concentrate the glycoproteins before glycan release. C18 stationary phase are used to retain residual amounts of proteins [33-35,37,38]. Amine, amide and graphitized carbon media can be used to retain glycans for further elution into solution [38-40].

Sample concentration for glycan detection is necessary which is alternatively done by vacuum centrifugation, lyophilization or bubbling dry nitrogen gas through the sample [28,29,32,41]. The derivatization of glycan is done before MS analysis as derivatization increases sensitivity and decreases fragmentation, this is particularly important for fucosylated and sialylated glycan because soft ionization source of electrospray and less soft MALDI can do their fragmentation [42]. Furthermore derivatization prevents the pre-fractionation by solid phase extraction. The abundance of sialylated glycans in human serum can suppress less abundant component such as high mannose glycans. The solid phase extraction methods are applied to concentrate high mannose glycan from the mixture by using varying ratio of water and acetonitrile which can be better visualized by mass spectrometry. Several method are applied to stabilize sialic acid to improve glycan stability and ion stability in mass spectrometer, these methods mainly includes methylation, permethylation and pyridylamination, these methods also have benefit of positive ion detection of sialylated glycans concurrently with neutral glycans [30,32-36,42]. The liquid chromatography can be used to monitor oligosaccharide, whose reducing end can be incorporated by fluorescent tags or by stable isotopes that enables relative quantitation [28,38-40,42].

Mass Spectrometry and Glycan Mass Profiling

The complete mixtures of Glycan are analyzed by obtaining masses with little or no separation is called as Glycan Mass Profiling (GMP). The complete compositional profile with regard to number of sialic acids, fucose, hexose and N-acetyl hexosamine is produced by this method [43]. GMP cannot distinguish between isomeric species (compounds with identical mass and composition) whereas it provides analysis on basis of mass and, therefore, composition. Glycan mass profiling is usually performed with MALDI or ESI as ionization source with only crude method of glycan purification. Glycan mass profiling is done using a Matrix-Assisted Laser Desorption/Ionization Fourier Transform Ion Cyclotron Resonance (MALDI-FTICR) mass spectrometer equipped with 7.0T magnet and infrared multi-photon dissociation (IRMPD) for tandem mass spectrometry. The FTICR mass analyzer with its high resolution of 105-106 at full width with half maximum and high mass accuracy of less than 10 parts per million (ppm) on internal standard used to profile glycan using exact masses [43].

During particular case of cancer the extent of glycosylation and type of O-glycans and N-glycans changes [44,45]. The selection of biomarker discovery on the basis of O-glycan or N-glycan release is not the trival choice. Previous studies have shown profound change in O-glycan in case of cancer, however, limited study on ovarian and breast has shown increase in N-glycan level between disease and control, however, it was found that O-glycan was contaminated with large number of N-glycan even when release procedure was reported to produce predominantly O-glycan [46,47]. The composition and the structure were confirmed by tandem MS including CID and IRMPD. The mass profiling studies to release N-glycan and those N-linked glycans with trimannosyl core (Man3GlcNAc2) and complex type N-glycan containing sialic acid found predominantly in human serum were profile by using mass profiling studies[27,31] . For obtaining putative structure of N-glycome from the composition, a theoretical library of human serum N-glycome was developed, which is highly effective for automatically annotating mass spectrum with a low false positive rate [27].

Glycan Chromatographic Profiling

The identification of individual oligosaccharides (specific isomeric structure) may provide more robust glycan markers with higher specificity than composition alone, until now the focus on glycan marker discovery has involved glycan mass profiling. Changes in specific linkages have been attributed to some disease [48,49]. The number of isomer greatly surpasses the number of compositions, providing a significantly larger set number of potential glycan markers [31,35]. The use of nanoflow liquid chromatography and capillary electrophoresis coupled with mass spectrometry have proved highly useful to gain access to individual glycan and separation of isomers from the component [31,35,38]. The diversity of N-glycan from human serum without derivatization can be assessed by employing porous graphitized carbon as stationary phase. Complex N-linked glycan found in human serum can be profiled as they constitute about 96% of total glycan present in serum while remaining 4% compromise of hybrid, and high mannose type glycan [31]. The technique to separate and simultaneously analyze neutral and anionic N-glycan from human serum without derivatization is rapid and highly sensitive tools for disease biomarker discovery.

Glycomics and Mass Spectrometry

In order to determine whether glycosylation has been changed in disease state sample as compared to healthy control, the glycan are harvested by glycomics approach without the prior knowledge of associated proteome [4,26,46,50]. Advances in glycomics analysis rely on the analytical tool for glycan profiling. Mass spectrometry has developed a central tool for glycomics analysis; it is also a precise tool for structural elucidation resulting in significant progress towards the role of the glycome in many biological systems [51,52]. The search for biomarker discovery is becoming more refined as the methods for profiling oligosaccharide composition and structure have become more refined [31,53].

Mass spectrometers capable of analyzing glycan structure typically employ Matrix-Assisted Laser Desorption /Ionization (MALDI) or electrospray ionization (ESI) with or without chromatographic separation. The instrument with high mass accuracy such as modern Time- Of- Flight (TOF), Ion Cyclotron Resonance (ICR) and orbitrap mass analyzer are useful for glycan detection because they can detect low or sub ppm mass error for exact mass annotation. The instrument having low performance can make glycan analysis challenging due to false assignment and high uncertainties associated with their measurement [27]. The false assignment can be overcome by using Tandem MS which provide structural information and glycan composition. Structural elucidation can be obtained by using glycan fragments, exact mass and exoglycosidase digestion.

Lectin Microarray for Glyco-biomarker

Glycosylation is major form of posttranslational modification and alteration in glycosylation is associated with cell differentiation and malignant transformation [54-57]. This make it potential diagnostic indicator of cell differentiation and malignant transformation. The diversity of glycoform at cell surface and combinatorial possibilities inherent in glycan structure far exceed DNA- and peptide- based structural diversity [58]. The glycome which is biological significant due to structural diversity is also important in rapid detailed analysis. Lectins, which are glycan binding protein has long been used in techniques such as blot, flow cytometry and immunohistochemistry to characterize serum or cell by focusing on individual glycan [56-61]. The sugar isomers can be differentially discriminated by lectin based approaches [63]. The availability of large number of lectins with variety of glycan specificities, provide an alternative for structure analysis by using them in microarray to provide more accurate and detailed "cell surface glycan signatures". Lectin microarray has been used for immune cell differentiation, pathogen cell tropism and cancer-stem cell identification. Hsu and Coworker used 21 lectin microarrays to distinguish O-antigen on Lipopolysaccharides (LPS) of bacterial cell surfaces, further the lectin microarray were used to capture glycosylation- defective cell lines [64]. The key features of infectious pathogen is their ability to directly bind to cell surface receptor via adhesin, the vast majority of adhesin are lectin. Which bind with carbohydrate moieties on cell surface and it help to trace the pathogen tropism and host-pathogen interaction [65].

The glycan-lectin interaction is relatively weak in comparison with antigen-antibody interaction. The problem of scanning of lectin microarray through scanner is weak signal, because some glycan dissociate from glycan-lectin during washing process [66]. Unfortunately most conventional microarray scanner requires the washing process. Hirabayashi and coworker has developed a unique lectin microarray based on the principle of evanescent-field fluorescence detection, the sensitivity of the array platform is up to 10pg of protein for assay [66]. During the case of carcinogenesis and oncogenesis the gene expression pattern of individual glycosyl-tranferases alter which correlates with the changes in glycosylation. Therefore it is possible to identify cell surface glycan by means of differential profiling, owing to its high sensitivity and accuracy, lectin microarray is best tool for selection of cancer –specific lectins and for quality control of stem cell before transplantation [65,67-69].

Prospect of Glycan Markers

The change is serum of cancer patients has been well identified through changes in glycosylation which is derived from cancer induced chronic inflammation. Glyco-biomarkers are indirect marker of tumor [2]. The amount of cancer derived glyco-biomarkers make very small percentage of total serum glycome, approximately, 40 g/L of glycoprotein are present in the serum of the total 3L of plasma in circulation. The low levels of glyco-biomarkers are identified by the advent of new technologies which allow the analysis of serum glycoprotein directly from 2D-PAGE, which identifies tumor specific biomarker such as gp73, PSA and RNAse I which have altered glycosylation during cancer [70-73]. The level of SLeX have been implicated for the diagnosis of cancer, the level of SLeX have been low for initial stages of non small cell lung cancer (21% for all stages), however, this increases to 71% for late stages cancer. The sensitivity of mucin biomarker CA15-3 is 61.5% for detecting metastatic breast cancer and the level of SLeX has increased to 78.5% [74]. The serum marker for inflammation is CRP which can be used for diagnosis of cancer, the serum level of CRP increases during acute and chronic inflammation. Serum level of CRP may pre-diagnose the onset of cancer as the level of CRP above baseline may be linked to develop the risk of colon cancer [2]. Several researchers have shown the role of N-glycan and O-glycan as disease markers [4,26,46,51]. The changes in N-glycan at the level of sialylation and fucosylation have been reported in many diseases [43,75]. During the disease the truncation of O-glycan and changes

in branching of N-glycan, acetylation, branching and expression of sialic acids have been linked to disease [75]. In prostrate cancer the difference in fucosylation and mannosylation has been found variable during the disease [26,34,78]. In breast cancer the increase in intensity of high mannose N-glycan and fucosylation in O-glycan has been reported [34,79,80]. In liver cancer the level of fucosylation has been elevated either in core or outer arm, and same can be observed in pancreatic and ovarian cancer [81].

Glycomics in Clinical Studies

The single-dot tissue glycome profiling incorporated with lectin- assisted fractionation increases the differential analysis of un-fucosylated/α2,6-sialylated glycoproteins in tissue samples from colorectal cancer and improved the quality of glycoproteomics in prognosis of disease [82]. The usage of liquid chromatography and mass spectrometry (LC-MS/MS) revealed the change in expression of 54 glycoproteins expressed differentially in hepatocellular carcinoma patient and healthy controls. These proteins are supposed to be involved in several biological processes, cellular components and molecular functions of hepatocarcinogenesis. Several of them had been reported abnormally regulated in several kinds of malignant tumors, and may be promising biomarkers of hepatocellular carcinoma [83]. Comparative glycoproteomics have identified a glycoprotein that was altered in both amount and glycosylation as a function of liver cirrhosis. The altered glycoprotein was an agalactosylated (G0) immunoglobulin G molecule (IgG) that recognizes the heterophilic alpha-gal epitope. As bacterial infection was a major complication in patients with cirrhosis and bacterial products such as LPS are thought to play a major role in the development and progression of liver fibrosis, this finding has many clinical implications in the etiology, prognosis and treatment of liver disease [84]. Recent progress in development of lectin-assisted glycan profiling lead to the development of sandwich assay kit for monitoring liver fibrosis in patients with viral hepatitis [85]. The usage of glycoproteomics has provided alpha-1-antitrypsin (SERPINA1) and apolipoprotein A-I (APOA1) as potential biomarker and therapeutic target for hepatocellular carcinoma (HCC) [86]. The differentiation in ovarian tumors was done by application of glycoproteomics techniques and the identified glycoproteins for histological subtypes of ovarian tumor will facilitate the understanding of molecular basis, diagnosis of ovarian tumor subtypes and prediction for treatment responses to therapeutic agents [87]. Glycans stand out from all classes of biomolecules because of their unsurpassed structural complexity, which is generated by variability in anomeric status of the glycosidic bond and its linkage points, ring size, potential for branching and introduction of diverse site-specific substitutions. The challenge for analytical processing of glycan which form the basis for the fingerprint-like glycomic profiles of glycoconjugates and cells. The sensitivity of the glycosylation machinery to disease manifestations, earning glycan assembly a reputation as a promising candidate to identify new biomarkers.

Future Challenges in Glyco-Biomarker Discovery

The goal of biomarker discovery is to increase the sensitivity and specificity that are useful for clinical diagnosis. It is quite difficult to identify true cancer glyco-biomarkers from complicated mixture of serum of cancer patient that contain complicated protein patterns that are not related to cancer cells [88,89]. The biomarker for cancer patients involves the analysis of serum at advanced stages without paying much attention to histopathological status [90]. The marker that can differentiate between healthy individuals and patient with advanced stage of cancer but the useful marker make up less than 1% of differential markers identified [90]. In case of liver cancer the weight of average normal liver weight about 1.5-2.0 kg while the tumor is only about 1.0 to 1.5 cm in diameter, which is about 1% of whole liver weight, thus the cancer derived glycoprotein in which glycan structure is altered from that of non-cancerous cells constitute less than 1% of glycoprotein population [88]. The challenge for future research is to increase the sensitivity of assay for biomarker for early detection; only 1 μg of glycoprotein is required for early detection by MS technology, thus it is difficult to identify glyco-biomarker in serum using MS technology [89]. The need of detection technology with higher sensitivity need to develop which can detect marker in one-tenth of cancer tissues currently needed [90]. The binding affinity of lectins is quite weak which is inappropriate for sensitivity detection of glycans, to find the proper solution to this problem two possible ways, one to find antibodies that recognizes specific glycan structure and second is the amplification of lectin signals that result from lectin binding to increase their sensitivity [66]. The feasibility of using biomarker in drug development process is indispensable, the use of biomarker in phase II clinical trial studies have widely accepted to improve drug development [89]. The tools of proteomics and glycomics have failed to produce marker that have clinical endpoint due to lack of specificity [90]. The metabolic oligosaccharide engineering is another area which involves the imaging of glycome in vivo by imaging cell-surface glycan, expression, internalization and trafficking in real time [91-93]. Various approaches by using azido-functionalized sugars are developing which serve as covalent attachment site for imaging probes and are installed into glycan using cell metabolic machinery [91]. The use of click chemistry based in vivo imaging has been used to visualize cell surface glycan because fluorescent probes are impermeable to cell membrane, these techniques are evolving for understanding proteoglycan structure diversity and changes during disease and can help to find new glyco-biomarker for disease and drug discovery.

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