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Invited Mini Review

Pros and cons of using aberrant glycosylation as companion biomarkers for therapeutics in cancer

*Jeong-Gu Kang, Jeong-Heon Ko & Yong-Sam Kim**
Division of KRIBB Strategy Projects, KRIBB, Deajeon 305-806, Korea

Cancer treatment has been stratified by companion biomarker tests that serve to provide information on the genetic status of cancer patients and to identify patients who can be expected to respond to a given treatment. This stratification guarantees better efficiency and safety during treatment. Cancer patients, however, marginally benefit from the current companion biomarker-aided treatment regimens, presumably because companion biomarker tests are dependent solely on the mutation status of several genes *status quo*. In the true sense of the term, "personalized medicine", cancer patients are deemed to be identified individually by their molecular signatures, which are not necessarily confined to genetic mutations. Glycosylation is tremendously dynamic and shows alterations in cancer. Evidence is accumulating that aberrant glycosylation contributes to the development and progression of cancer, holding the promise for use of glycosylation status as a companion biomarker in cancer treatment. There are, however, several challenges derived from the lack of a reliable detection system for aberrant glycosylation, and a limited library of aberrant glycosylation. The challenges should be addressed if glycosylation status is to be used as a companion biomarker in cancer treatment and contribute to the fulfillment of personalized medicine. [BMB reports 2011; 44(12): 765-771]

INTRODUCTION

Prevention and treatment have been the main approaches adopted to improve health and, they serve as a double-edged sword in the biomedical realm. We have seen tremendous progress in both areas for the last a few decades, and an increased understanding of molecular events occurring inside diseased organisms has inarguably led to improvements in medical science. However, we still have a long way to go before we have comprehensive and efficient therapeutic options, particularly for obstinate diseases such as cancer, diabetes mellitus, etc. It is a reality that a majority of deaths still originate from cancer, stroke, and cardiovascular disease (1).

To address these challenges, increased efforts have been being made to enhance the efficiency of treatments, including combining treatment agents with diagnostics, which opened the era of 'theragnostics (therapeutics plus diagnostics)'. This approach is based not only on the notion that every genetic identity has its suitable method of treatment, but also on the idea that the current treatment regimes can be replaced with molecular-targeted therapy. Especially, gene-based in vitro diagnostic (IVD) testing boasts unsurpassed increments in growth rate, with thousands of genes being targeted for new molecular in vitro tests (2). These IVD tests are comingled with the therapeutics in the pipeline of drug development, thereby implementing the 'right treatment to the right patient' strategy and producing better clinical outcomes. Nonetheless, current medications with IVDs are used in a dichotomic fashion; genetic mutation of a gene is the sole criterion for the choice of a treatment and one of the bisected groups is intended to benefit from the therapeutic approach. Because every patient identified as belonging to the 'supposed to benefit' group does not respond to the treatment, the current therapeutic strategy does not meet the goal of personalized medicine. To fulfill this ultimate goal in cancer, we need to know every possible molecular signature and environmental factor governing the efficiency and efficacy of a treatment.

As a case in point, we suggest in this review that the glycosylation status of a molecule can be a critical determinant for therapeutic choices in cancer. We introduce evidence that glycosylation variants are observed in cancer and affect the development and progression of diseases. In addition, we highlight the promising and limiting aspects associated with the use of glycosylation status as a companion biomarker for therapeutics.

Brief history of targeted therapy

Cancer is the leading cause of death worldwide and a tremendous effort has been made to develop anti-cancer drugs. Contrary to traditional chemotherapy which is usually intended to interfere with rapidly dividing cells, the currently used drugs are targeted at specific molecules required for tumorigenesis and proliferation, and thereby guarantee improvements in both efficacy and safety. A brief review of the history of molecular-targeted therapy in cancer will be presented to provide deeper insights for understanding companion biomarker-guided targeted

*Corresponding author. Tel: +82-42-860-4156; Fax: +82-42-879-8119; E-mail: omsys1@kribb.re.kr
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therapies which will be discussed.

Imatinib mesylate (Gleevec, also known as STI-571) is regarded as the first success story in molecular-targeted therapy. This drug specifically inhibits ABL-BCR tyrosine kinase activity and is effective in the treatment of chronic myeloid leukemia (CML) patients who have the 'Philadelphia' chromosome (3-5). Rationale underlying the development of imatinib mesylate contributed to the design of ensuing kinase inhibitors and monoclonal antibodies for cancer treatment. The long development time required from the identification of the 'Philadelphia chromosome until the approval of Gleevec was significantly shortened for the development of the ensuing tyrosine kinase inhibitors. Herceptin, which targets the Her2/neu tyrosine kinase receptor (also known as ErbB2) overexpressed in some types of breast cancer, mirrors the progress in recent drug development (6, 7). In this case, the accompanying diagnostic test for HER2 expression, known as the HercepTest, provided the treatment stratification by enabling physicians to identify the patients who are considered to benefit from the monoclonal antibody (8). Similarly, gefitinib (Iressa, also known as ZD1839) was intended to target epithelial growth factor receptor (EGFR), which is overexpressed in non-small cell lung cancer and other solid tumors including colon and breast cancer (9). The efforts to identify target molecules to control cancer have led to an expanded list of target molecules, including not only various kinase receptors such as vascular endothelial growth factor receptor (10) and ALK (11), but also non-kinase molecules, ie., bcl-2 (12), PARP (13), estrogen receptor (14), Janus kinase (15), and PI3K (16).

Progress in DNA sequencing and microarray techniques have made it possible to compare genome-wide studies on the relation of genetic variations with diseases. In genome-wide association studies (GWASs), whole genes were subjected to analysis for the association of person-to-person gene variation and diseases, leading to, for example, the discovery of strong associations of the deletions close to the gene encoding complement factor H (CFH), complement factor H receptor 1 (CFHR1) and CFHR3 with a reduced risk for age-related macular degeneration (17). Currently, 4,000 SNP associations are claimed for $\geq$200 diseases in $\geq$1,200 human GWASs (18). Along with genomic studies, we have equipped ourselves with top-notch proteomics and systems biology-based techniques to expand the spectrum of genes and proteins 'targetable' for diseases.

Companion biomarker as part of the drug development

The mutation study on the KRAS gene has opened a new era of targeted therapy, exemplifying the importance of discovering and testing an associated factor which affects responsiveness to a drug. After it had been reported that the HRAS gene shows a point mutation at codon 12 (19), similar mutations in KRAS and NRAS were reported (20, 21). Of note is the finding that the KRAS mutation status plays a critical role not as a drug target but as a predictive biomarker for tumor responsiveness to anti-EGFR monoclonal antibody therapies (22-24). Cetuximab and panitumumab are anti-EGFR drugs developed for the treatment of colorectal cancer and were found to produce a response only in KRAS mutation-negative patients. This case reflects the importance of a companion biomarker when devising a specific therapeutic regimen that is optimal for treatment based on the disease status of a particular patient and mirrors the future direction of diagnostics in the development of therapeutics. Directly or non-directly, the bio-molecular signature other than the target molecule will guide the treatment strategy, identify the right patients who will experience the best results in the response to a therapeutic agent, and help develop the best suited program of medication.

Pari passu with this direction, the Food and Drug Administration recently issued guidelines on 'Companion Dx' which support the development of innovative new targeted medicines and their corresponding diagnostic tests, and are intended to provide manufacturers with greater predictability (25). It is hoped that these guidelines will help the commercial therapeutic manufacturers develop the best suited drugs for responder populations and will spare non-responders from exposure to potential side effects of drugs that will not work for them.

There are, however, many obstacles to overcome to realize *bona fide* 'personalized medicine' with the aid of companion biomarkers in cancer. There are only a few groups into which patients can be partitioned based on available companion biomarkers. Referring to the case above, every KRAS mutation-negative patient is not responsive to a single anti-EGFR monoclonal antibody. This implies that patients with a disease should be classified into multiple groups by using multiple variables which evidently affect the pathological processes of a disease. In this sense, fulfillment of personalized medicine requires that classification of patients should be supported by sufficient information on genetic mutations beyond what has currently been discovered, as well as by expression profiles, post-translational modifications (PTMs), and time-dependent variation of molecular signatures obtained from rigorous basic research.

Alterations in the glycosylation status associated with cancer

Immature proteins are enzymatically synthesized in the endoplasmic reticulum (ER), and later undergo decoration with one or more chemical moieties, termed post-translational modification (PTM). Glycosylation is typically one of several PTMs that are usually found in eukaryotic cells and produced by enzymatic catalysis, as opposed to non-enzymatic chemical reaction of glycation. Protein glycosylation can be classified into several types according to the glycan linkage site: N-linked glycosylation, O-linked glycosylation, and glycophosphatidylinositol (GPI)-anchored glycosylation (Fig. 1). N-linked glycosylation is formed at the asparagine (N) site of the N-X-S/T sequence where S and T represent serine and threonine, respectively, and X can be any amino acid except proline. Mature N-linked glycan consists of a core structure containing 2 *N*-acetylglucosamine and 3 mannose residues, of which 2 mannoses are elongated with antenna formed by galactose, N-acetylglucosamine, N-acetylgalactosamine, fucose, and sialic acid. O-linked glycosylation is a relatively late-stage

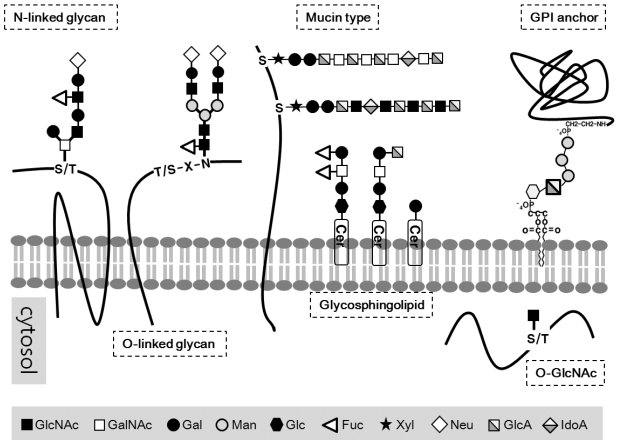

Fig. 1. Common glycosylation patterns in human cells. Secreted and transmembrane proteins can usually possess either N-linked or O-linked glycans, or both. GPI anchored protein and glycosphingolipids are located exclusively in membrane regions. O-GlcNAcylated proteins are usually found in the intracellular compartments, playing a role in modulating cellular signals. Note that glycosylation is usually not clonal and the glycan structures depicted for N-linked, O-linked, and glycosphingolipids illustrate one of the structural libraries.

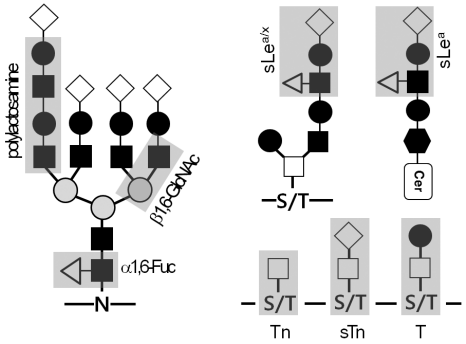

Fig. 2. Aberrant glycan structures commonly found in cancer. It is interesting to note that N-linked glycans are substrate for various glycosyltransferases and consequently become bulky in cancer cells, as opposed to O-linked glycans that are often truncated by glycosidases. Sialyl Lewis antigens are frequently observed at the terminal region of O-linked glycans and glycosphingolipids, although they can also be seen for N-linked glycans.

event in the protein maturation and often occurs with the attachment of glycans to serine and threonine, and, to a lesser extent, to hydroxyproline and hydroxylysine. O-linked glycans are formed in a stepwise fashion with sugars added incrementally. The most common type of O-glycosylation is observed in the 'mucin-type' glycan where the reducing terminal N-acetylgalactosamine (GalNAc) is added and further extended with galactose (Gal), N-acetylglucosamine (GlcNAc), and sialic acid. In addition to the mucin-type O-linked glycans, a variety of mammalian proteins are known to have mannose, fucose, galactose or glucose as reducing terminal linkages. Especially, simple O-linked glycan comprising a single GlcNAc residue is observed to play an important role in the modulation of the biological activity of intracellular proteins (26), often competing with phosphorylation (27). GPI anchored proteins are linked at their carboxyterminus through a phosphodiester linkage of phosphoethanolamine to a trimannosyl-non-acetylated glucosamine (Man3-GlcN) core. The reducing end of GlcN is linked to phosphatidylinositol which is then anchored by another phosphodiester linkage to the cell membrane through its hydrophobic region. The Man3-GlcN oligosaccharide core may undergo various modifications during secretion from the cell. GPI-anchored proteins also play a critical role in a variety of receptor-mediated signal transduction pathways, adhesion, and antigenicity (28). In addition to protein glycosylation, glycan is attached to sphingolipids, thus forming glycosphingolipids, which are generally called gangliosides. The glycan structures in glycoproteins and gangliosides have diverse roles in cell-cell recognition, molecular function and stability, and cell adhesion, etc (29).

A unique feature is that a glycoprotein shows heterogeneity in its glycan structure because it is synthesized using a non-template-driven biosynthetic process in endoplasmic reticulum and Golgi body, and a lack of any proofreading machinery. In addition, the structure of the biosynthetic end product is dependent on the polypeptide backbone as well as a number of variable factors such as the expression levels of glycosidases and glycosyltransferases and the availability of substrates, which fluctuate during cell growth, differentiation and development (30, 31). Aberrant glycosylation, which is frequently observed in tumors, occurs with the perturbed expression of responsible glycosyltransferases and can depend on the availability of substrate saccharides. Among the best characterized glycosyltransferases is N-acetylglucosaminyltransferase V (GnT-V), which is overexpressed through regulation by the Ets-1 transcription factor in malignant cancer cells (32), and sialyltransferases (33), which are at least partly responsible for the generation of the polylactosamine residues, polysialic acid or some gangliosides. The sialylated glycan structures including sialyl Lewis antigen (sLe) and sialyl Tn (sTn) are also observed on glycoproteins and gangliosides in various cancer, which are frequently associated with poor prognosis in patients with breast (34), colon (35), stomach (36) cancer. Importantly, the presence of truncated O-glycans at the surface is a common feature in cancer, and consequently, antigenic peptide backbones are exposed (37). Glycosylation changes that are frequently observed in cancer are summarized in Fig. 2.

Aberrant glycosylation is a functional marker that can be used to gauge the clinic-pathogenic process in cancer. This notion is well illustrated by the functional role of the aberrant tissue inhibitor of metalloproteinase-1 (TIMP-1) in cancer invasion and metastasis (38). TIMP-1 is an endogenous glycoprotein which inhibits several matrix metalloproteinases (MMPs) in a 1 : 1 stoichiometric manner, and is known to regulate cancer metastasis. In opposition to the previous reports on their relative avail-

ability, we proposed that TIMP-1 is aberrantly glycosylated by initiation of GnT-V, and the aberrant TIMP-1 fails to tightly bind gelatinases possibly due to electrostatic repulsion and steric hindrances generated by newly attached bulky glycans. The mitigated affinity toward gelatinases is responsible for the loss of gelatinase inhibition by TIMP-1, resulting in enhanced cancer invasive/metastatic potential of colon cancer cells. Lectin blot analysis following immunoprecipitation of TIMP-1 in colon tumors pointed to the involvement of aberrant TIMP-1 in cancer progression. Mostly found in O-linked glycoproteins and gangliosides, sialyl Lewis structures on tumor cells also function as a biomarker for the malignant potential of a tumor. They are recognized by specific endogenous lectins (E-selectin) on endothelial cells and play an important role as the binding ligand for the lectin, allowing cancer cells to maintain firm adhesion in the presence of shearing forces in the early phase of extravassation during cancer metastasis (39, 40). Perhaps an inspiration could be extracted from the previous studies on the functional effects of aberrant glycosylation in cancer; while many of the glycan structures themselves are not tumor-specific, the appearance of those structures on a specific protein can be a useful biomarker that provides a molecular history of the development and progression of cancer and thereby contributes to guiding molecularly targeted therapeutic options.

Promises and limitations in the use of aberrant glycosylation as companion biomarker

In spite of tremendous progress in glycobiology and glycomics research, we are just at a primitive stage for predicting who will respond to a drug by examining the glycol-pattern or a specific glycan structure of a glycoprotein. To my knowledge, there is no direct evidence that glycosylation structure(s) is a reference that may be helpful to define appropriate and timely treatments. Although fucosylated AFP (AFP-L3) is upregulated in liver cancer, little is known about how the molecular function of AFP-L3 may contribute to the development or progression of cancer. Any utilization of a companion biomarker, contrary to diagnostic or monitoring biomarkers, should be evidence-based.

Despite the lack of relevant evidence and information *status quo*, we can find some clues suggesting that the behavior of therapeutic agents can vary with the glycosylation status of particular molecules. First, the transportation of a drug can be affected by the glycan structure of a transporter responsible for delivery into cells or any cellular compartments. Case in point, Chen et al. raised a relevant issue in their study, in which they suggested that mature N-linked glycans of the UT-A1 urea transporter are essential to UT-Al activity by contributing to the UT-A1 trafficking into membrane lipid raft subdomains (41). This implies that the premature or aberrant glycans may be responsible for the malfunction of the transporter activity. Many of the membrane transporters, including P-glycoprotein, are glycoproteins and the identification of relevant drug transporters and their glycan status need to be monitored. Second, glycosylation status can affect 'drug resistance'. ABC transporter is a glycoprotein that plays a role in the efflux of various drug and organic cationic or neutral compounds (42). Many lines of evidence indicate that ABC transporter is responsible for multi-drug resistance via affecting the efflux of drugs. Although the glycomic nature of ABC transporter family members has not been fully elucidated, we are close to being able to say that the glyco-profiles of ABC transporters change with disease status and that altered glycosylation may affect the efflux of anti-cancer drugs. We can find a link between defective glycosylation of the ABC transporter and drug resistance from a recent study. Beretta et al. suggested that patients with oxaliplatin-refractory ovarian carcinomas may benefit from non-Pt-based regimens which do not contain resistance-associated protein (MRP) 1 and MRP4 substrates, showing that altered glycosylation of MRP1 is found in cells selected for resistance to the Pt drugs (43). Because the ABC transporter family consists of a large number of transporter members, a systemic approach should be taken to identify transporters responsible for the efflux of a particular drug and to define glyco-moieties affecting their activities. Third, the glycan status of a drug target protein may indirectly affect the interaction with biologics. No protein molecule behaves alone, and instead dynamically interacts with a library of binding partners. The nature of molecular interactions usually belongs to one of the protein-protein, protein-nucleotide, protein-lipid, or protein-carbohydrate binding, although other interactions may exist. As expected, protein-carbohydrate interactions are most affected by the glycosylation status. Lectins comprise a group of proteins that bind to the glycan portion of biomolecules and are divided into several types depending on their structures and the nature of interaction. Galectin is a very specialized lectin that shows an affinity for galactose-containing glycans (44-46). There are 14 family members reported in human, each of which plays a distinct role in adhesion, differentiation, growth, apoptosis, etc. Galectin-1 alone makes up a complicated interaction network with plasma membrane proteins and peptidoglycans in the extracellular matrix (46-48). Because most monoclonal antibody-based anti-cancer drugs target plasma membrane proteins (e.g. EGFR), they are thought to be in a constant competition with other binding partners such as galectin-1. The altered glycan structure of a target molecule for an anti-cancer drug may hinder or reinforce the interactions with lectin-type molecules, which may in turn, affect the pharmacodynamic and pharmacokinetic properties of monoclonal antibody-based anti-cancer biologics. These possibilities await a definitive study on which target shows a different glycan pattern that is dependent on disease state, how the alteration affects the interaction with any possible binding partner, and whether the perturbed interaction leads to changes in pharmacodynamics and pharmacokinetics of therapeutics.

In spite of these potentials mentioned above, there are several limitations that may hamper the development of glyco-pattern(s) as companion cancer biomarkers. Most of these limitations derive from the lack of assay methods required to monitor glycosyl-alterations with sufficient analytical and clinical validity. A majority of the monitoring of glycosyl-alteration has been per-

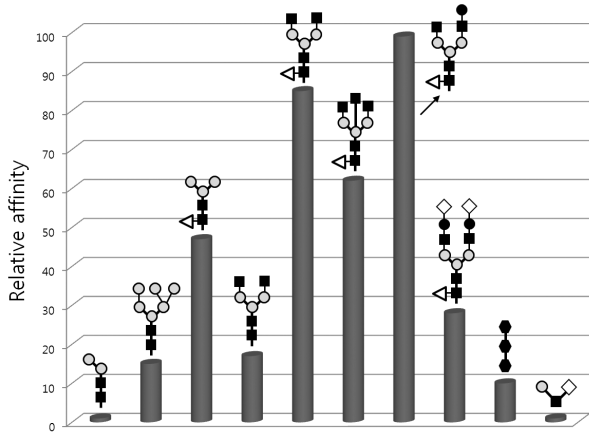

Fig. 3. Poor selectivity of *Lens culinaris* (LCA) lectin in glycan recognition. LCA binds, but not exclusively, to fucosylated N-glycans, and has group specificity toward glycans with similar structures. Together with relatively low affinity compared to antibodies, this low selectivity of lectins may hamper their use as a probe for subtle changes in glycan moieties in clinical settings.

formed by the use of lectin as probe (49). Despite the specificity and affinity toward glycans, there are limitations when lectins are used for clinical purposes. For instance, *Lens Culinaris* (LCA) is a lectin that is often used to monitor the fucosylated N-glycans in experimental settings. The Lectin Frontier Database (http://riodb.ibase.aist.go.jp/rcmg/glycodb/LectinSearch), however, shows that LCA exhibits a broad specificity for fucosylated as well as non-fucosylated glycans, albeit a difference in the binding strength among the glycans (Fig. 3), and this low specificity is a common characteristic for most of lectins. Moreover, most commonly used lectins do not have a high affinity toward their binding glycan partners and show a dissociation constant (K_d) value in the range of 10^{-7}-10^{-5} M except for a few toxin-ganglioside interactions (50), for which K_d values are lower than those of most antibodies. The low sensitivity observed especially when monitoring the glycans of sparse candidate biomarkers is attributable to the low affinity of lectins. These obstacles may be overcome by attempts to develop glycan-specific antibodies with sufficient specificity and affinity (51). Finally, the glycan library that has been found to exist in nature is not expansive enough to personally classify every patient by using the glycan alone. This may imply that a glycan must be used as an auxiliary biomarker in combination with other biomarkers. A recent finding that glycans are decorated by phosphorylation (52) is quite interesting in the sense that glycans can be further modified by another chemical moiety and accordingly, broaden the glycan library that can be used as companion biomarkers for given therapeutics.

CONCLUSION

It is generally accepted that future therapeutic approaches will be more molecular-targeted and guided by evidence-based biomarkers. The direction toward theragnostics will eventually lead to personalized medicine in cancer, dramatically promoting the efficiency of treatment. This implies that every possible companion biomarker that can help guide the therapeutic options should be developed to identify and classify patients according to their potential responsiveness to specific drugs. Evidence is accumulating that the dynamism of glycan structures reflects the disease state in cancer, and some glycans are used to detect and monitor cancer. Evidence-based, unbiased information must be accumulated in advance for the glycan dynamism to be used as a companion biomarker in clinical settings, and thereby contribute to personalized medicine.

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