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Alterations in glycosylation as biomarkers for cancer detection

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ABSTRACT
Glycoconjugates constitute a major class of biomolecules which include glycoproteins, glycosphingolipids and proteoglycans. Glycans are involved in several physiological and pathological conditions, such as host—pathogen interactions, cell differentiation, migration, tumour invasion and metastisation, cell trafficking and signalling. Cancer is associated with glycosylation alterations in glycoproteins and glycolipids. This review describes various aspects of protein glycosylation with the focus on alterations associated with human cancer. The application of these glycosylation modifications as biomarkers for cancer detection in tumour tissues and serological assays is summarised.

INTRODUCTION
Glycosylation is a common post-translational modification of proteins, and variation in oligosaccharide structures is associated with many normal and pathological events: host—pathogen interactions, differentiation, migration, tumour invasion and metastisation, cell trafficking and signalling. Cancer is associated with aberrations in glycolipids and glycoproteins.[1 2] In glycoproteins, about half of which are glycosylated in eukaryotes, both *N*-glycans and *O*-glycans can be synthesised, and both can be affected during cancer progression. *N*-glycans have a functional role in cell adhesion, and modifications in cancer cells are associated with invasion and metastisation.[3] O-Glycosylation of glycoproteins, of which mucin glycoproteins are a major component because of their high content of serine and threonine and the fact that they are highly overexpressed in carcinomas, contributes to a substantial part of cancer biomarkers and will be the focus of the present review. The review is directed to non-specialised scientific readers assumed to be familiar with cancer nomenclature and concepts. It is intended to give an overview of the normal process of glycosylation and the alterations associated with cancer and their usefulness as tumour markers.

GLYCOSYLATION IN HUMAN CELLS
Glycosylation is the covalent attachment of a carbohydrate to a protein, lipid, carbohydrate or other organic compound, catalysed by glycosyltransferases, using specific sugar donor substrates. Glycans are found in several types of biomolecule which can be classified into different families of glycoconjugates: glycoproteins, glycosphingolipids, proteoglycans and glycosylphosphatidylinositol-linked proteins (figure 1).

As mentioned above, there are two types of glycan in glycoproteins: *N*-glycans and *O*-glycans. Both types of glycosylation often coexist in the same protein and in the same cell. N-Glycosylation consists of an oligosaccharide chain N-linked to asparagine in the sequence context Asn-X-Ser/Thr, where X is any amino acid except proline. In rare cases, the sequence Asn-X-Cys is also used. N-Glycosylation requires the production of an oligosaccharide precursor which is transferred en bloc to nascent proteins in the endoplasmic reticulum (ER). After the transfer of the oligosaccharide precursor structure to the nascent protein, several subsequent processing reactions occur in the ER, including cycles of glucose removal and addition, which contribute to protein folding. In addition, *N*-glycan chains can be further diversified in the Golgi apparatus, with terminal saccharide residues.

O-Glycosylation is the other type of glycosylation found in glycoproteins and consists of a glycan O-linked to a serine or a threonine residue (figure 2). The frequency of O-glycosylation on glycoproteins is high, particularly on secreted or membrane-bound mucins, which are rich in serine and threonine. The first step in mucin-type O-glycosylation is the transfer of GalNAc from a sugar donor UDP-GalNAc to serine and threonine residues and is controlled by UDPGalNAc-polypeptide *N*-acetylgalactosaminyltransferases (ppGalNAc-Ts).[4–6] To date, more than 15 distinct members of the mammalian ppGalNAc-T family have been identified and characterised,[7–20] and in silico analysis indicates that as many as 20 ppGalNAc-Ts may exist.[6] They control the first level of complexity of mucin glycosylation—that is, the sites and density of *O*-glycan occupancy of the mucin tandem repeat. This is because ppGalNAc-Ts, although catalysing the same enzymatic step, display different tissue expression specificity[21 22] and have different kinetic properties and acceptor substrate specificities.[5 11 23] This enzymatic specificity may lead to different functions depending on the cell type and organ in which it is expressed.[9 10 24–26] Altered expression of ppGalNAc-Ts may be one of the mechanisms involved in changes in mucin O-glycosylation during malignant transformation.[21 22 27–30]

A second level of complexity in mucin O-glycosylation is the processing of carbohydrate chains by other glycosyltransferases. After the first glycan (GalNAc) is added forming the Tn antigen, the core 1 structure is synthesised by Gal-transferase (C1GalT-1), which adds Gal to GalNAc, forming the core 1 (T antigen). Alternatively, Tn and T antigens can be sialylated by sialyltransferases forming the sialyl-Tn, sialyl-T and disialyl-T

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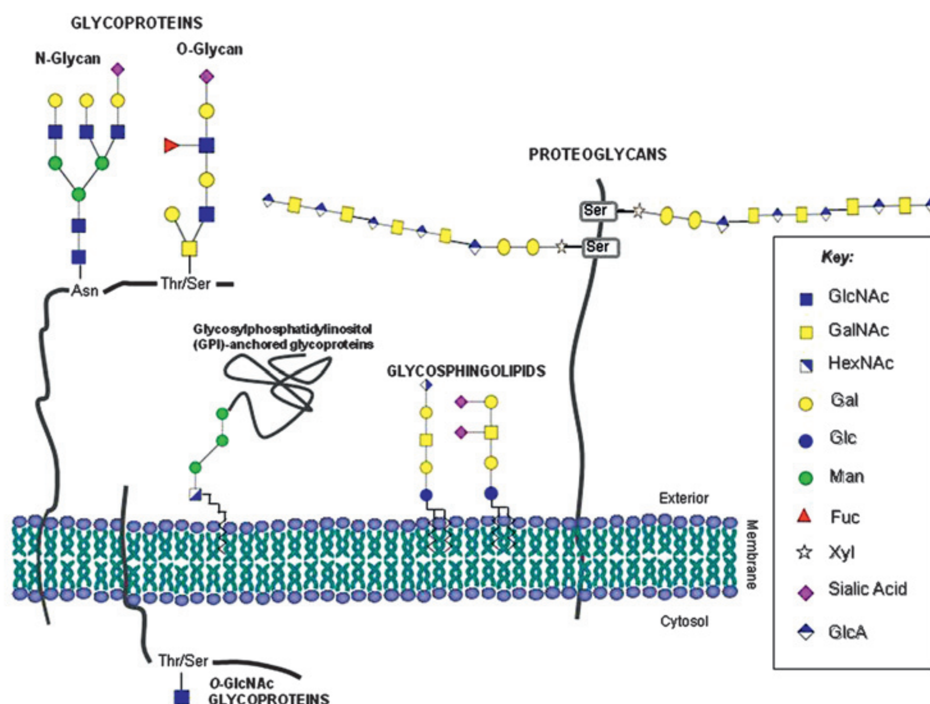

Figure 1 Schematic representation of common classes of glycoconjugates expressed in human cells. Protein O-glycosylation and N-glycosylation can occur in both membrane-associated and secreted glycoproteins.

Figure 2 Schematic representation of the biosynthetic pathways of most common mucin-type *O*-glycans. Glycosyltransferases involved in the enzymatic steps are indicated. The major tumour-associated antigens are highlighted. b3Gal-T, β1-3-galactosyltransferase; b4Gal-T, β1-4-galactosyltransferase; b3Gn-T, β1,3-N-acetylglucosaminyltransferase; C1Gal-T1, core 1 β1-3-galactosyltransferase; C2GnT, core 2 β1—6 *N*-acetylglucosaminyltransferase; ppGalNAc-T, UDPGalNAc-polypeptide *N*-acetylgalactosaminyltransferase; ST3Gal, α2,3-sialyltransferase; ST6GalNAc-I, GalNAc α2,6-sialyltransferase.

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antigens.[31–34] Formation of the sialyl-Tn antigen stops any further processing of the oligosaccharide chain[31 32 35] (figure 2).

Another common core structure present in normal cells contains a branching GlcNAc attached to core 1 and is termed core 2 (figure 2).[36] Core 2 is produced in many epithelial and haematopoietic cells. The enzyme responsible for core 2 synthesis is core 2 β1–6 *N*-acetylglucosaminyltransferase (C2GnT).[37] At least three genes encode this subfamily (C2GnT1 to C2GnT3) of a larger family of β1–6 *N*-acetylglucosaminyltransferases.[38] There are two major types of C2GnTs. The L type (leucocyte type, C2GnT1 and C2GnT3) synthesises only the core 2 structure, whereas the M type (mucin type, C2GnT2) is also involved in the synthesis of core 4 and other GlcNAcβ1–6-linked branches (figure 2). The C2GnT1 and C2GnT3 enzymes are active in many tissues and cell types, but the C2GnT2 enzyme is found only in mucin-secreting cell types.[38 39] The expression and activity of C2GnTs are altered in certain tumours. Because of their branched nature, core 2 *O*-glycans can block the exposure of mucin peptide epitopes. There are other types of core structures, and most of them show tissue specificity expression.

The extension of the core structures is catalysed by β3/4 Gal-Ts and β3/4 Gn-Ts (figure 2) 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 mucins as in other glycoconjugates. The families of glycosyltransferases that catalyse the addition of these terminal structures are described in detail below. In contrast with *N*-glycans, *O*-glycans do not have sialic acid α2-6Gal linkages, although the sialic acid α2-6GalNAc moiety is common, for example, in the sialyl-Tn antigen. Thus, in most mammalian mucin-producing cells, α2–6 sialyltransferases act on GalNAc, and α2–3 sialyltransferases act on galactose. Some of the sialyltransferases prefer *O*-glycans as their substrate, but many of these enzymes have an overlapping specificity and also act on *N*-glycan structures as acceptor substrates. There are other types of non-mucin *O*-glycans, such as *O*-GlcNAc in proteins found in the cytoplasm and nucleus; these are not the focus of this review.

Another important group of glycoconjugates are proteoglycans. These consist of a core protein and covalently attached glycosaminoglycan chains, which are linear polysaccharides (figure 1). Proteoglycans are expressed in a tissue-specific manner and have been shown to participate in several cellular and extracellular interactions.

ALTERATIONS IN GLYCOPROTEIN O-GLYCOSYLATION IN CANCER AND PRENEOPLASTIC LESIONS

Altered glycosylation of cell surface glycolipids, membrane-associated glycoproteins and secreted glycoproteins is a quasi-universal modification in cancer.[40] This was first demonstrated by showing that antibodies raised against cancer cells often recognise abnormal glycan structures.[41] Despite there being no evidence for a role of altered glycosylation in cancer initiation, and despite information on the mechanisms that generate abnormal glycosylation still being limited, it is well established that it can contribute from early stages to invasion and metastisation.[2 42–44]

We will focus on glycan alterations of glycoproteins, in particular mucins that are major carriers of glycan structures in carcinomas, characterised by O-glycosylation initiated by addition of a GalNAc on serine or threonine residues. Of the various changes, the two most important ones from the standpoint of biomarker signatures are generation of truncated versions of normal oligosaccharides and generation of unusual forms of terminal structures, namely sialylated versions of the normal counterparts (figure 2). Most modifications are generated by upregulation/downregulation of glycosyltransferases, and one study has implicated a mutation in the Cosmc chaperone protein as the underlying mechanism for the absence of a functional enzyme, leading to accumulation of cancer-associated precursors.[45] Therefore it is reasonable to assume that the glycan signature at the cancer cell surface is unstable, at variance with what happens to most cancer-associated alterations, which are clonal because of their genetic origin. This fits into the mosaicism of glycan expression in tissue sections, reflecting variations in differentiation along cancer progression.[46] However, despite their non-clonal nature, it is clear that they stabilise during cancer progression,[47] probably because of the positive selective properties they confer on the cell populations, by facilitating invasion and metastisation.

It is relevant in considering altered glycosylation central to the cancer biomarker field that it is visible on the cell surface of cancer cells (and therefore easily accessible to antibodies or lectins as tissue biomarkers) and often expressed in the circulation, either on secreted products or by shedding from cell surfaces (and therefore identifiable as serum biomarkers).

As mentioned above, mucins are major carriers of cancer-associated carbohydrates and they amplify alterations at the surface of the cancer cell because they are highly overexpressed in cancer and have repetitive sequences rich in serine and threonine, the potential O-glycosylation sites.[48] They are major carriers of the modified glycans secreted or anchored at cancer cell membranes. However, they can themselves be biomarkers due to modifications induced by altered glycosylation. Such an example is the differential recognition of the MUC1 mucin by different monoclonal antibodies according to glycosylation.[49] Mucins can be either secreted or membrane-bound and contain both O-linked and N-linked oligosaccharides and share a common structural feature, the presence of a tandem repeat (VNTR) domain.[50–59] Tandem repeats are rich in serine and threonine, which can be O-glycosylated. The polymorphic nature of mucins at the VNTR was first recognised in 1987[50] and later shown, for the MUC1 mucin, to have implications for the risk of gastric cancer development,[60] partly by modulating glycosylation.[61] Mucins show restricted, tissue-specific, expression in normal epithelial cells, but are overexpressed and aberrantly expressed in cancer. As an example, MUC2 mucin, which is expressed in normal intestine, can be aberrantly expressed in intestinal metaplasia, a precursor lesion of gastric carcinoma,[62] and in 25% of gastric carcinomas.[63] Also MUC4 is expressed in premalignant and malignant lesions of the pancreas despite it not being expressed in normal pancreatic cells.[64] In some cases, modifications of mucin expression are strictly linked to modified glycosylation. For example, MUC2 mucin in intestinal metaplasia colocalises with expression of sialyl-Tn in goblet cells.[47 65 66] Future studies should address the putative coordination of mucin/glycosyltransferase regulation to clarify if, at least to some extent, mucin expression can 'instruct' glycosylation or if they are independently but coordinately regulated.

SIMPLE MUCIN-TYPE CARBOHYDRATE ANTIGENS

One of the most common cancer-associated modifications is poor glycosylation of glycoproteins, leading to expression of truncated *O*-glycans at the cell surface[67–69] (figure 1). These are Tn, sialyl-Tn and T antigens (figure 2), which are pan-carcinoma antigens.[70 71] Many studies have identified aberrant expression

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of these so-called simple mucin-type antigens in carcinomas from breast,[72] oesophagus,[73] colon,[74] pancreas,[75 76] stomach,[47] lung,[77] endometrium,[78 79] ovary[80] and bladder.[81] Other studies have shown an association of expression of these antigens with poor prognosis in patients with breast,[82] colon[83] and stomach[84 85] carcinoma.

Modifications stem from disorganisation of secretory pathway organelles (ER and Golgi) in cancer cells and altered glycosyltransferase expression. As mentioned above, these modifications can also occasionally depend on mutations in a chaperone essential for glycosyltransferase function.[45] Another, more common, mechanism for cancer-associated expression of truncated *O*-glycans is absence of glycosyltransferases responsible for the synthesis of core structures used as substrates for chain elongation.[31] Alternatively, or in combination with the previous mechanism, is overexpression of sialyltransferases responsible for the synthesis of sialyl-Tn and sialyl-T antigens.[35 86] In fact, transfection of cells that do not express sialyl-Tn with the ST6GalNAc I (GalNAc α2,6-sialyltransferase) is induced to express sialyl-Tn,[33 87] and, in human breast cancer, expression of ST6GalNAc I colocalises with expression of sialyl-Tn.[34] Furthermore, induced expression of sialyl-Tn in cell lines increases their tumorigenicity.[88 89]

Future studies will be directed at identifying specific *O*-glycan/core-protein combinations to increase the specificity of the biomarkers for cancer detection. In the case of MUC1, it has been shown that specific glycopeptide combinations can generate antibodies with increased specificity in cancer reactivity.[90]

LEWIS CARBOHYDRATE ANTIGENS (BIOSYNTHESIS AND EXPRESSION)

Lewis-type blood group antigens, such as sialyl Lewis A (SLea) and sialyl Lewis X (SLex) (figure 3), are expressed in cancer cells, mimicking their normal expression on blood cells (monocytes and neutrophils) and also mimicking their potential for migration through binding to endothelial cell selectins. They are expressed on carbohydrate chains, type 1 and type 2, according to the linkage between the galactose residue and the GlcNAc residue, β1,3 and β1,4, respectively. The presence or absence of type 1 Lewis antigens in a given individual depends initially on the presence of active enzymes responsible for the addition of the fucose monosaccharide. The α1,2-fucosyltransferase, product of the secretor gene (Se), acts on the terminal galactose and produces the H type 1 structure which forms the substrate for the α1,4-fucosyltransferase, the product of the Lewis gene (Le), which synthesises the difucosylated Le^b antigen (figure 3). People with inactivating mutations of the Se gene are unable to synthesise H type 1 and Le^b antigen; they are called non-secretors and constitute 20% of the human population. The secretor and Lewis status of individuals are implicated in susceptibility to several diseases, mostly infections, with almost complete absence of gastrointestinal infections from calicivirus in non-secretors[91] and an implication of BabA+ *Helicobacter pylori* infection.[92]

The relevance of Lewis sialylated structures in cancer was first revealed in the 1980s, when monoclonal antibodies raised against cancer cells were shown to recognise $SLe^{a/x}$.[93–95] The biosynthetic basis for sialylated Lewis antigens also started to be

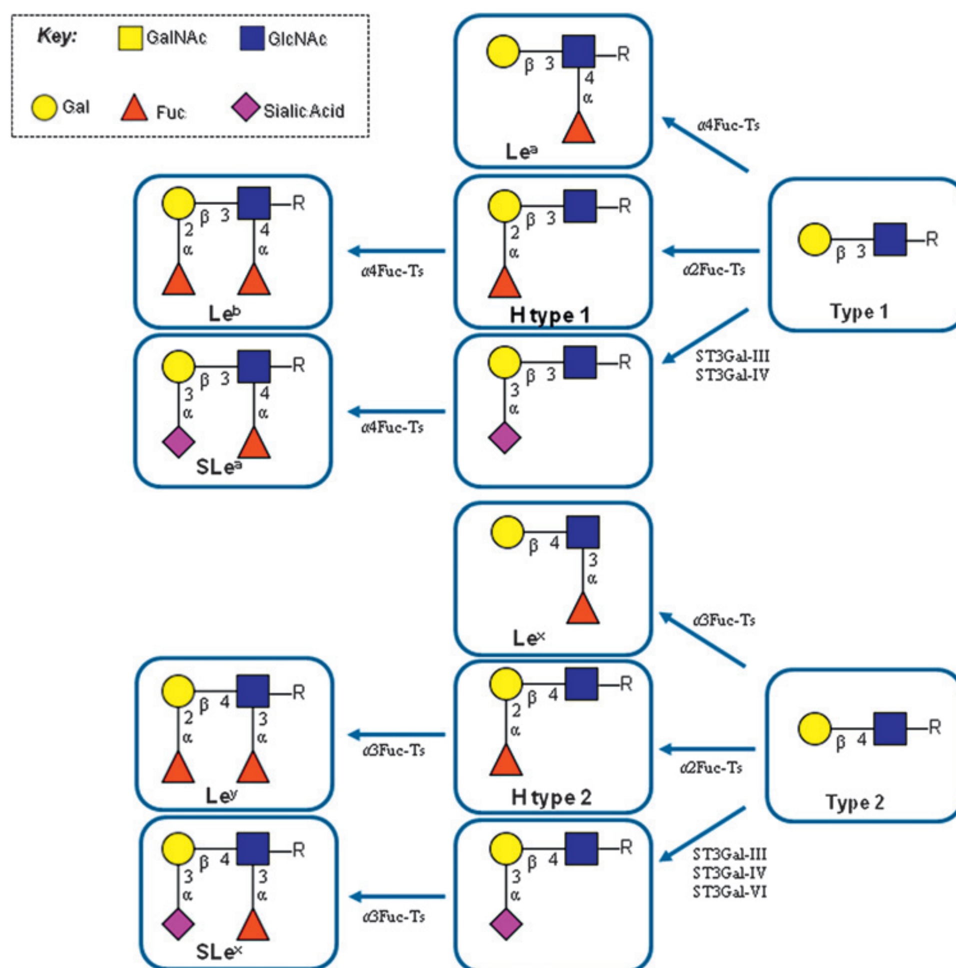

Figure 3 Schematic representation of the biosynthesis of Lewis antigens. R represents precursor carbohydrate chain. Fuc-T, fucosyltransferase; Le^a, Lewis A; Le^b, Lewis B; Le^x, Lewis X; Le^y, Lewis Y; SLe^a, sialyl Lewis A; SLe^x, sialyl Lewis X; ST3Gal, α2,3-sialyltransferase.

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determined in the 1980s,[96 97] and it is now recognised as depending on increased α2,3-sialyltransferase and/or α1,3/4-fucosyltransferase activities.[98 99] In leukaemias, a viral gene product of human T-cell lymphotropic virus type 1 trans-activates fucosyltransferase VII, an α1,3-fucosyltransferase with rate-limiting activity for the synthesis of sialyl-Le^x in leucocytes, and induces strong constitutive expression of sialyl-Le^x in leukaemic cells.[100]

Mucins can be carriers of these glycan structures,[101] and MUC1 was specifically identified as one such carrier.[102] In 1991, it was demonstrated that SLe^a and SLe^x were recognised by endothelial leucocyte adhesion molecule 1 (ELAM-1) in endothelial cells[103] and also that cancer cells use these structures to adhere to activated endothelial cells[104] and facilitate establishment of haematogenous dissemination and metastatisation. In agreement with this, overexpression of SLe^x and SLe^a is common in carcinomas of several origins (eg, lung, colon, gastric and pancreas) and is associated with increased metastatic ability[105–108] and poor survival of the patients.[109–113]

The relevance of SLe^a and SLe^x to cancer dissemination led to attempts to use them not only as cancer biomarkers but also as therapeutic targets. One therapeutic strategy is to reduce synthesis of SLe^x by using competitive disaccharide substrates as decoys.[114] Antisense strategies, directed to α1,3/4-fucosyltransferase, were successful in reducing liver metastisation in a mouse model.[115] Similarly, increased and cancer-associated expression of these antigens has been used for in vivo bioimaging.[116]

Mechanisms controlling gene expression, including methylation and identification of transcription factors, are under investigation to better understand aberrant expression of Lewis antigens in cancer and to improve their usefulness as cancer biomarkers.

GLYCAN-BASED SEROLOGICAL ASSAYS IN CANCER

Glycosylation changes on glycoconjugates either present on the surface or secreted by cancer cells are a major potential source of cancer biomarkers. At present, most serological assays used for cancer detection, prognosis and monitoring are based on quantifying glycoconjugates in the serum of patients with cancer. These serological assays detect carbohydrate antigens such as SLe^a (CA19-9) and STn (CA72-4) or mucin glycoproteins such as MUC1 (CA15-3) and MUC16 (CA125).[57 117–119]

The use of these biomarkers for cancer screening is limited because of their broad expression by various types of cancer, precluding identification of the organ in which the cancer has originated.[120–122] In addition, these biomarkers can also be produced in some non-neoplastic and inflammatory diseases,[123] reducing the specificity of the assays for screening purposes.[124] Nevertheless, sound data support the use of the CA125 assay for detection of ovarian cancer. Raised CA125 concentrations are found in 50% of patients with stage I ovarian cancer and in 25% of serum samples collected 5 years before diagnosis of ovarian cancer.[125]

In general, the detection of these biomarkers in the serum of patients with cancer has been shown to be particularly useful for evaluation of prognosis and for monitoring purposes. This is the case for the assay of CA125, which is detected in 80% of patients with ovarian cancer[117]; furthermore, increases and decreases in CA125 correlate with regression and progression of the disease. In addition, preoperative evaluation of CA125 has been shown to aid evaluation of prognosis for patients with ovarian cancer.[126 127]

Similarly, the aberrantly glycosylated MUC1 mucin, which is produced by cancer cells and shed into the circulation, can be detected by the CA15-3 assay. Raised CA15-3 concentrations have been shown to be useful for prognosis evaluation in early-stage breast cancer and for monitoring the course of the disease,[128–130] including monitoring patients with metastatic disease during active therapy.[131] In the absence of readily measurable disease, an increasing CA15-3 concentration may indicate treatment failure.[124 132] Evaluation of the clinical utility of CA15-3 in other cancers is under investigation.

The aberrant expression of other carbohydrate antigens on glycoconjugates has also been shown to be useful for evaluating prognosis and for monitoring purposes in cancer. Serological detection of SLe^a on glycolipids and glycoproteins by the CA19-9 assay has been performed in patients with an established diagnosis of pancreatic, colorectal, gastric or biliary cancer and used to monitor clinical response to therapy.[133 134] In addition, in colon cancer, a raised CA19-9 concentration before surgery has independent prognostic value: patients with increased concentrations had a fourfold increase in death rate at 3 years compared with those with lower concentrations. In gastric carcinoma, preoperative CA19-9 concentration remains one of the best prognostic factors,[135 136] and preoperative positivity for CA19-9 is an independent risk factor for recurrence of gastric carcinoma.[137]

Another carbohydrate antigen, sialyl-Tn, which is expressed in glycoproteins such as mucins, can be detected by the CA72-4 assay. Raised CA72-4 concentration has been shown in patients with gastric, colorectal and pancreatic carcinomas.[136 138] In gastric carcinoma, CA72-4 has been shown to be useful as an independent prognostic factor: patients positive for CA72-4 show a 3.8-fold higher risk of death.[139] The CA72-4 assay is also useful for monitoring gastric carcinoma, where positivity is considered to be a predictor of tumour recurrence.[136] CA72-4 has also been shown to be an independent prognostic factor in pancreatic cancer.[140]

Determination of carcinoembryonic antigen (CEA) is another serological assay widely used in clinics. CEA glycoproteins are rich in *N*-glycans, and these glycoproteins are produced by normal and carcinoma cells. In colorectal and some other cancers, CEA is expressed at high level and shed into the circulation.[129–131] The clinical significance of serum CEA in patients with colorectal carcinoma is in the evaluation of prognosis and follow-up of patients.[141 142] Increases in serum concentrations of CEA can also have non-cancer-related causes.[140] Because of its lack of sensitivity in the early stages of colorectal cancer, CEA measurement is unsuitable for population screening.

The glycosylation alterations observed in cancer, particularly the putative glycopeptide specificities been identified above, constitute a major target for the development of novel serological-based assays for early cancer detection with major screening and clinical implications.

Take-home messages

- Glycoconjugate modifications are a quasi-universal hallmark of cancer which makes them important cancer biomarkers.
- Some modified glycoconjugates detected in serum are in clinical use for follow-up of patients.
- New developments are expected to increase the scope of their clinical application, particularly at the diagnostic level.

PERSPECTIVES

Glycoconjugate modifications are a universal hallmark of cancer, which makes them important cancer biomarkers. Many

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of the current biomarkers used in clinics, in both tissue and serum assays, are based on these carbohydrate modifications. Their basis and precise structure are, however, largely not understood by those who use them in the clinical setting. This stems from the molecular complexity of the expression of these biomarkers and the still largely unknown regulatory pathways. Given the importance of these biomarkers because of both their high frequency and high accessibility at the cell surface and in serum, this review is an attempt to give the clinical/pathological expert a relevant biochemical understanding of the field.

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Alterations in glycosylation as biomarkers for cancer detection

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