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Monoclonal Antibodies Against Tumour-Associated Carbohydrate Antigens

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Abstract

Glycomic profiling of tumour tissues consistently shows alterations in N- and O-glycosylation profiles of glycoproteins and glycolipids compared to healthy tissues, with important functional implications for cancer cell biology. The overexpression of tumour-associated carbohydrate antigens (TACAs), as a result of aberrant glycosylation in tumours, is usually correlated with poor prognosis and survival of cancer patients. In tumours, TACAs are associated with worse tumour progression than the deletion and inactivation of tumour suppressor genes. The findings of TACAs acting are not merely tumour markers but also constitute part of the machinery in inducing cancer metastasis and invasiveness further strengthen the scientific rationales for immunotherapy targeting TACAs. Despite the attractiveness of the TACAs, there are very few anti-glycan monoclonal antibodies (mAbs), as glycans usually induce low-affinity IgM responses. This chapter provides an overview of TACAs, direct killing anti-glycan mAbs, and introduces two murine mAbs (FG88 mAbs) that recognise Lewis carbohydrate antigens overexpressed on tumour glycoconjugates with high functional affinity. Although the production of anti-glycan mAbs against cancers is not new, the production of high-affinity IgG anti-glycan mAbs is novel. FG88 mAbs definitely have great potential in cancer therapy and serve as valuable tools in glycobiology research.

Keywords: cancer, Lewis carbohydrate antigen, therapeutic monoclonal antibody, oncosis, antibody drug conjugate

1. Introduction

Cell surface glycosylation is a post-translational modification of proteins and lipids, which is universal to all living cells and plays an important role in cell signalling, immune recognition and cell-cell interactions [1]. Monosaccharide units serve as building blocks of glycans

Figure 1. Common glycoconjugates on human cells.

(polysaccharides) that are synthesised by a complex series of post-translational enzymatic steps [1, 2]. There are several main families of glycoconjugates: (1) the Asn-linked (N-linked) and Ser-/Thr-linked (O-linked) oligosaccharides that are present on many glycoproteins, (2) the glycosaminoglycans (GAGs) either as linear-free polysaccharides (such as hyaluronan) or attached to serine residues of proteoglycans (such as heparin sulphate and chondroitin sulphate), (3) the sphingolipids that consist of oligosaccharides linked to ceramide and (4) the glycosylphosphatidylinositol (GPI)-linked proteins, which are proteins that express a glycan chain linked to phosphatidylinositol [2, 3] (Figure 1).

2. Glycoproteins

It has been appreciated for some time that protein glycosylation is the most complicated post-translational modification that a protein can undergo [4]. Protein glycosylation is important as it alters the behaviour of proteins, making them more soluble, protecting them from proteolysis, covering antigenic sites and altering the orientation of proteins on cell surfaces [5]. In glycoproteins, the carbohydrate units are linked to the protein backbone by N- and/or O-glycosidic bonds, C-mannosyl bonds, phosphoglycosyl bonds and glypiated linkage (GPI anchor) [4].

N-glycans are covalently attached to the asparagine (Asn) residues of proteins, and the consensus sequence for N-glycosylation is Asn-X-Ser/Thr, where X can be any amino acid except proline. In O-glycosylation, the glycan is attached to the side chains of serine or threonine residues. Unlike N-linked glycosylation, no consensus sequence defining an O-linked glycosylation site has been reported in Ref. [4]. C-mannosylation is a novel type of protein glycosylation, which differs fundamentally from N- and O-glycosylations. It involves covalently

attachment of an α -mannopyranosyl residue to the indole C2 carbon atom of tryptophan (Trp) via a C-C link [6, 7]. The phosphoglycosyl bond is another distinct type of glycopeptide linkage [N-acetylglucosamine (GlcNac), mannose (Man) and fucose (Fuc)] involving an attachment of a carbohydrate to protein via a phosphodiester bond [8]. Another important carbohydrate-protein connection is the GPI anchor. In this connection, mannose is linked to phosphoethanolamine, which in turn is attached to the terminal carboxyl group of the protein [9].

3. Glycolipids

Glycolipids constitute approximately 3% of the outer layer of the plasma membrane, and they are composed of a lipid tail and a carbohydrate head. Glycolipids are classified into three main groups, including glycosylglycerolipids, glycosylphosphatidylinositols (GPI) and glycosphingolipids (GSLs), based on the type of lipid component. Of these glycolipids, GSLs are the ones that are most widely overexpressed on tumours [10].

GSLs are ubiquitous membrane constituents, which are embedded in the cell plasma membrane [11]. Ninety percent of mammalian GSL biosynthesis begins with the synthesis of glucosylceramide (GlcCer), which is a key precursor of the glycosphingolipid series [12]. The process takes place on the cytosolic face of the Golgi complex via the action of the Type I transmembrane protein glucosylceramide (GlcCer) synthase [13], which transfers a glucose (Glu) residue to ceramide in the β -glycosidic linkage [14, 15]. Galactose is then added to GlcCer to generate lactosylceramide (LacCer) by β -1,4-galactosyltransferases in the lumen of the Golgi apparatus [10]. Further glycosylation steps are catalysed by different glycosyltransferases

Figure 2. Synthetic pathways for the major GSL species. Lactosylceramide provides the branch point for different GSL series.

with different specificities and result in the generation of more complex GSLs [16]. The major GSL series are defined by their internal core carbohydrate sequence. They are the ganglio-series (galNac β 1-4 gal), globo-series (gal α 1-4 gal), lacto-series (gal β 1-3glcNac β 1-3 gal) and neolacto-series (gal β 1-4glcNac β 1-3 gal). LacCer provides the branch point for the synthesis of all these GSL series (**Figure 2**) [12].

4. Aberrant glycosylation in cancers

Aberrant glycosylation has been described as one of the hallmarks of cancer. Aberrant glycosylation of proteins and lipids during malignant transformation leads to the overexpression of tumour-associated carbohydrate antigens (TACAs) [17]. Evidence is accumulating that TACAs have contributed to various aspects of cancer development and progression, including proliferation, invasion, angiogenesis and metastasis [2, 18]. Thus, studying the mechanisms and consequences of variations in glycosylation associated with cancers will provide crucial insight into cancer progression. Importantly, TACAs are overexpressed mostly on tumour cell surface, making them potential diagnostic markers and ideal therapeutic targets [19].

In general, aberrant glycosylation in cancers is due to the following changes: (1) under- or overexpression of glycosyltransferases, (2) altered expression of glycoconjugate acceptor [20], (3) altered sugar nucleotide transporter activity [21] and (4) improper function of the Golgi structure [22].

4.1. Altered glycosylation patterns in cancer

In cancers, the expression of glycosyltransferases is often deregulated. For example, N-acetylglucosaminyltransferase V (GnT-V), which catalyses the formation of β -1,6-GlcNAc branching structures, is expressed only at very low level in normal mammary gland. However, in cancer, the expression of GnT-V has been upregulated, resulting in highly branched N-glycan structures which were found to be associated with cancer growth and metastasis [20, 23–25]. Overexpression of beta-1,3-N-acetylglucosaminyltransferase 8 (β 3GnT8) results in increased levels of polylactosamine structures in colorectal carcinoma [26], and upregulation of N-acetylglucosaminyltransferase III (GnT-III) increase bisected N-glycans in liver cancer [27, 28]. Aberrant expression of alpha-N-acetylgalactosaminide alpha-2,6-sialyltransferase 1 (ST6GalNAC-I) in breast cancer resulted in the sialylation of Tn antigen to form the sialyl-Tn (STn) antigen [29]. Altered expression of fucosyltransferases is responsible for the aberrant expression of Lewis carbohydrate antigens such as Lewis a (Le^a), sialyl-Lewis a (SLe^a), Lewis x (Le^x) and Sialyl-Lewis x (SLe^x) in many types of tumours [30–32].

Incomplete glycosylation is another abnormal feature found in human cancer. The expression of truncated O-glycans such as Tn, STn and T antigens had been reported in a wide range of tumours [33–36]. Incomplete glycosylation of these truncated glycans is due to the defects in the secretory pathway organelles (endoplasmic reticulum and Golgi) [33], the absence of glycosyltransferases responsible for the generation of core glycan for chain elongation [37] and the overexpression of sialyltransferases responsible for the addition of terminal sialic acid

(i.e. conversion of Tn to STn antigen) [33, 38, 39]. For example, in neoplastic cells, alterations in glycosylation of O-linked glycans had been shown to affect oligomerisation of cell surface receptors, thus influencing the stimulation of these receptors. Wagner et al. demonstrated the inhibition of complex O-glycan formation (N-acetyl-galactosamine galactose core I structure and its subsequent sialylation), resulted in the impairment of death receptors 4 and 5 (DR4 and DR5), which significantly impact the apoptotic pathway signalling by TNF-related apoptosis-inducing ligand (TRAIL) [40].

In addition, specific changes in O-glycan (GalNAc-Ser/Thr) and N-glycan core structures have been reported to lead to the generation of different core glycans with different degrees of glycan branching [27, 28], which in turn significantly impact the overall glycan structure and function.

4.2. Tumour-associated carbohydrate antigens (TACAs)

4.2.1. Altered sialic acid expression

As early as the 1960s, there was evidence that tumour cells of various origins increased the expression of sialic acids on membrane glycoproteins and glycolipids as well as their secretion into the tumour microenvironment [41–43]. Sialic acids on normal cells are involved in multiple different physiologic processes [44]. However, hypersialylation of tumour cells specifically benefits tumour cell growth, promotes metastases [45, 46] and correlates with a poor prognosis of cancer patients [47].

Sialic acids are nine-carbon backbone α -ketoacidic sugars [3]. In general, sialic acids terminate the outer end of glycans (sialoglycans) via more than 20 distinct Golgi-resident sialyltransferases (ST). This enzymatic process is carried out via their second carbon (C2) to either galactose (α 2-3Gal or α 2-6Gal), N-acetylgalactosamine (α 2-6GalNAc) or another sialic acid (α 2-8Sia) [48]. Altogether, the different linkages to underlying sugars result in a tremendous diversity of sialoglycans [44]. Sialoglycans are known to participate in cell-cell and cell-extracellular matrix interaction, including adhesion, migration and immune recognition [44]. Sialic acid-binding immunoglobulin-like lectins (Siglecs) are receptor families that specifically recognise sialoglycans. Siglecs can be found on most immune cells, and they can transmit immunosuppressive signals upon binding to sialic acid ligands. Thus, increased expression of siglec ligands by tumour cells could contribute to tumour immune invasion [49].

There are a number of causes of the increase in cell surface sialic acid [50]. Changes to the core structures of N-glycans are one of the most common aberrant glycosylations in cancer. Increased activity of GNT-V (also known as MGAT5) was found to result in larger and more branched N-glycans, thus providing additional acceptors for terminal sialylation [20, 50]. Similarly, carcinomas that overproduce mucins (heavily glycosylated high-molecular-weight glycoproteins, e.g. MUC1 and MUC4) which contain aberrant O-linked glycosylation can lead to increased sialylation [51, 52]. Together with increased expression of sialyltransferases [53], these enzymes increase cell surface sialylation and metastatic potential [20]. In addition, tumour cells often overexpress α 2-6 sialic acid, mainly due to upregulation of the ST6Gal-I [54–56] or ST6GalNAc sialyltransferases [29, 57] that respectively conjugate terminal sialic

acid to N-glycans or O-glycans and glycolipids [53]. Mass spectrometry analysis of human serum sialo-glycoproteins revealed increased expression level of α 2-6 sialylation in breast cancer [58] and lung cancer samples [59], whereas α 2-3 sialylation was increased in prostate cancer samples [60], malignant brain tumours [61] and ovarian serous carcinomas [62].

Aberrant expression of sialic acids confers major advantages to tumour cells. Therefore, by targeting these, sialoglycans overexpressed on tumours may be highly beneficial.

4.2.2. *Altered Lewis carbohydrate antigen expression*

Lewis carbohydrate antigens can be found on various glycoconjugates in most human epithelial tissues [63]. They are formed by the sequential addition of fucose onto oligosaccharide precursor chains on glycoproteins or glycolipids through the action of a set of glycosyltransferases [64]. Le^x was reported to be overexpressed in breast and gastrointestinal carcinomas. Normal expression of Le^x is restricted on certain normal epithelial cells including the oesophagus, stomach, small bowel, ciliated epithelium of trachea, bronchus [65, 66] and normal human polymorphonuclear neutrophils (PMNs) [67]. Le^y was reported to be overexpressed on ovarian, breast, prostate, colon and lung carcinomas. Although Le^y expression can be found on both normal and neoplastic tissues, Le^y distribution differs between the two tissue types. Expression of Le^y on normal epithelial tissues is restricted to the secretory borders of epithelial surfaces, making it less accessible to circulating antibodies. Conversely, Le^y expression on epithelial cancer cells occurs on all surfaces including luminal surfaces [65].

Sialylated Lewis carbohydrate antigens such as SLe^a and SLe^x are significantly enhanced in cancer [68, 69]. The expression of these cancer-associated antigens results mainly from the upregulation of sialyltransferases [68]. SLe^a is normally present on the inner surface of the ductal epithelium of a variety of epithelial tissues, which makes it largely inaccessible to antibodies and immune effector cells [70]. SLe^x can be found on granulocytes, normal oral mucosa and breast tissue [71]. Both SLe^a and SLe^x are found to be aberrantly expressed on the surface of a broad range of carcinomas such as breast, ovarian, melanoma, colon, liver, lung and prostate [72]. Overexpression of SLe^x and SLe^a appears to directly correlate with increased metastatic disease and poorer overall survival in patients with colorectal cancer invasion [73]. Similar results were obtained from analysis the combination of SLe^a, SLe^x and Le^y antigens in non-small-cell lung cancer (NSCLC) patients [74].

4.2.3. *Altered ganglioside expression*

Gangliosides are acidic glycosphingolipids with the presence of at least one sialic acid linked to their oligosaccharide chain [75]. Biosynthesis of gangliosides involves sequential addition of sialic acids to lactosylceramide (LacCer) by ST3Gal V (GM3 synthase), ST8Sial I (GD3 synthase) and ST8Sia V (GT3 synthase) that leads to the generation of the a-, b-, and c-series ganglioside precursors, respectively, representing the mono-, di-, and tri-sialylated gangliosides (**Figure 3**) [68]. In general, gangliosides are involved in cell-cell recognition or regulation of downstream signalling of various proteins (e.g. insulin, epidermal growth factor and vascular endothelial growth factor receptors) [76, 77].

Figure 3. Biosynthesis of gangliosides.

Tumour-associated gangliosides have been suggested as a result of initial oncogenic transformation and play a key role in the induction of invasion and metastasis [75, 78]. Examples of gangliosides that are overexpressed in cancers are GD2 in neuroblastoma [79] and small cell lung cancer (SCLC) [80], GD3 in melanoma [81], GM2 [82] and fucosyl-GM1 in SCLC [81].

Some gangliosides overexpressed in cancers have been identified as adhesion molecules, which promote tumour cell metastasis. Gangliosides such as GD2, GD3 and GT1b form complexes with integrins [83] in melanoma where the terminal sialic acid residues of these gangliosides inhibit cell attachment by abrogating the interaction between integrin $\alpha 5 \beta 1$ and fibronectin [84]. Gangliosides on tumour cells also promote metastasis by forming aggregates with peripheral blood mononuclear cells (PBMCs) or platelets. This is due to the interaction between sialic acid moieties on gangliosides with sialic acid-binding proteins named Siglecs (sialic acid/immunoglobulin/lectin) [85], which are expressed on various types of blood cells [86]. These tumour aggregates may induce the blood cells to release factors that activate endothelial cells to elicit cell adhesion molecules (ICAMs, VCAMs, E-selectin and P-selectin), which in turn initiate tumour cell adhesion or invasion [87, 88]. Siglec-7 has been reported to bind preferentially to sialyl-2 $\rightarrow$ 6 GalNAc [86]. Interestingly, GD3, GD2 and GT1b share the same sialyl-2 $\rightarrow$ 6 GalNAc moiety [86], and they are overexpressed on a variety of tumours [89]. Thus, disialo epitopes may promote metastasis by binding to Siglecs expressed on blood cells.

In addition to the cell adhesion function, gangliosides have been reported to act as immune checkpoint molecules to aid in the escape of tumour cells from immune surveillance. Gangliosides released from the active secretion of tumour cells into serum can be taken up by T cells, with ensuing inhibition of T-cell proliferation and activation. The inhibitory action includes the defects in antigen presentation and reduction of cytokine [IFN- γ], interleukin-2 (IL-2) and IL-4] production [90–95]. The molecular mechanism of ganglioside-induced T-cell dysfunction was suggested to involve the inhibition of NF- κ B (NF- κ B) activity of T cells via degradation of RelA/p50 dimer and p50/p50 homodimer proteins [96, 97].

4.3. Serum cancer biomarkers

Better survival rates among cancer patients are correlated with earlier detection. The utilisation of serum cancer biomarkers has played a major role in not only early detection of cancer but also prediction of cancer recurrence following initial therapy [98]. However, current clinically approved serum cancer biomarkers are characterised by low sensitivity in detecting cancers [99]. Thus, the development of highly sensitive novel serum cancer biomarkers with better diagnostic and prognostic performance may enhance early detection rates and identification of new targets for anticancer therapy.

4.3.1. α -Fetoprotein (AFP)

α -Fetoprotein (AFP) is a 70 kDa glycoprotein [99], normally only secreted by foetal liver and present in foetal serum [100]. However, under certain pathological conditions, when it is present in adult serum, AFP is associated with cancer. Thus, AFP has been used as a serological marker for the early diagnosis of hepatocellular carcinoma (HCC) [101] and non-seminomatous germ cell tumours (NSGCT) [102].

AFP has a single N-linked oligosaccharide with a biantennary complex-type structure, with altered core fucosylation and terminal sialylation in HCC and NSGCT (**Figure 4**) [102]. Kobayashi et al. reported higher α -1,6-fucosyltransferase (FUT8) expression in HCC tissues than non-cancerous tissues and increased in fucosylation were correlated with HCC

Structure	Glycan
	$\begin{array}{l} \text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \\ \text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \end{array} \rightarrow \begin{array}{c} 6 \\ \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc} \\ 3 \end{array} \rightarrow \begin{array}{c} \text{Fuca}\alpha 1 \\ \downarrow 6 \\ 4\text{GlcNAc} \end{array}$
	$\begin{array}{l} \text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \\ \text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \end{array} \rightarrow \begin{array}{c} 6 \\ \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc} \\ 3 \end{array} \rightarrow \begin{array}{c} \text{Fuca}\alpha 1 \\ \downarrow 6 \\ 4\text{GlcNAc} \end{array}$
	$\begin{array}{l} \text{SA}\alpha 2 \rightarrow \text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \\ \text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \end{array} \rightarrow \begin{array}{c} 6 \\ \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc} \\ 3 \end{array} \rightarrow \begin{array}{c} \text{Fuca}\alpha 1 \\ \downarrow 6 \\ 4\text{GlcNAc} \end{array}$
	$\begin{array}{l} \text{SA}\alpha 2 \rightarrow \text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \\ \text{SA}\alpha 2 \rightarrow \text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \end{array} \rightarrow \begin{array}{c} 6 \\ \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc} \\ 3 \end{array} \rightarrow \begin{array}{c} \text{Fuca}\alpha 1 \\ \downarrow 6 \\ 4\text{GlcNAc} \end{array}$
	$\begin{array}{l} \text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \\ \text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \end{array} \rightarrow \begin{array}{c} 6 \\ \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc} \\ 3 \end{array} \rightarrow \begin{array}{c} \text{Fuca}\alpha 1 \\ \downarrow 6 \\ 4\text{GlcNAc} \end{array}$
	$\begin{array}{l} \text{SA}\alpha 2 \rightarrow \text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \\ \text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \end{array} \rightarrow \begin{array}{c} 6 \\ \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc} \\ 3 \end{array} \rightarrow \begin{array}{c} \text{Fuca}\alpha 1 \\ \downarrow 6 \\ 4\text{GlcNAc} \end{array}$

Figure 4. The structures of the N-linked glycans expressed on AFP associated with HCC and NSGCT patients.

progression [101]. In addition to HCC, overexpression of FUT8 in thyroid carcinoma tissue has been linked directly to tumour size and lymph node metastasis [103]. In a study, Osumi and coworkers demonstrated that upregulated of FUT8 expression in cancer cell regulated the expression of E-cadherin. E-cadherin was responsible in the enhancement of cell-cell adhesion, which in turns contributes to the metastatic potential of cancer cells [104].

4.3.2. Prostate-specific antigen (PSA)

Prostate-specific antigen (PSA) is a 28.4 kDa glycoprotein with an N-linked glycosylation site. PSA has been used widely to screen for prostate cancer in men [99]. PSA is normally secreted by the prostate epithelium and periurethral glands. In prostate cancer, disruption of the prostate epithelium leads to the release of PSA into serum [99]. When compared to PSA isolated from healthy individuals, PSA isolated from the serum of prostate cancer patients shown

Structure	Glycan
FA2G2	$\begin{array}{l} \text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \rightarrow \begin{array}{c} 6 \\ \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc} \\ 3 \end{array} \\ \text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \rightarrow \begin{array}{c} 6 \\ \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc} \\ 3 \end{array} \end{array}$ $\text{Fuca}1 \downarrow 6$
FA2(6)G1S1	$\begin{array}{l} \text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \rightarrow \begin{array}{c} 6 \\ \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc} \\ 3 \end{array} \\ \text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \rightarrow \begin{array}{c} 6 \\ \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc} \\ 3 \end{array} \end{array}$ $\text{Fuca}1 \downarrow 6$
FA2(6)BG1S1	$\begin{array}{l} \text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \rightarrow \begin{array}{c} \text{GlcNAc}\alpha \\ 1 \downarrow 4 \\ 6 \\ \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc} \\ 3 \end{array} \\ \text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \rightarrow \begin{array}{c} 6 \\ \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc} \\ 3 \end{array} \end{array}$ $\text{Fuca}1 \downarrow 6$
A2	$\begin{array}{l} \text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \rightarrow \begin{array}{c} 6 \\ \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc} \\ 3 \end{array} \\ \text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \rightarrow \begin{array}{c} 6 \\ \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc} \\ 3 \end{array} \end{array}$
A2G2	$\begin{array}{l} \text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \rightarrow \begin{array}{c} 6 \\ \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc} \\ 3 \end{array} \\ \text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \rightarrow \begin{array}{c} 6 \\ \text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc} \\ 3 \end{array} \end{array}$
<div> Fucose (Fuc)</div> <div> N-acetylglucosamine (GlcNAc)</div> <div> Mannose (Man)</div> <div> Galactose (Gal)</div>	

Figure 5. N-linked oligosaccharide structures of PSA elevated in prostate cancer patient serum.

significant higher levels of core-fucosylated biantennary glycans and α -(2,3)-linked sialic acids [105–109]. PSA is composed of several glycoforms [110, 111]. PSA with core-fucosylated biantennary glycans (FA2G2, FA2(6)G1S1 and FA2(6)BG1S1) and terminal α -(2,3)-linked sialic acids (A2 and A2G2) (**Figure 5**) were found elevated in prostate cancer patient serum [60].

4.3.3. Cancer antigen 19-9 (CA19-9)

Cancer antigen 19-9 (CA19-9) corresponds to a carbohydrate structure, sialyl-Lewis a (SLe^a) [99], which is overexpressed on cancer cell surface as a glycolipid and/or as an O-linked glycoprotein [112]. CA19-9 was first characterised by 1116-NS19-9 mAb [113] and has been found primarily in pancreatic and biliary tract cancers [113]. It has been used as a serum bio-marker for pancreatic cancer [114]. In neoplastic tissues, epigenetic silencing of the gene for

α -(2,6)-sialyl transferase leads to the abnormal synthesis and accumulation of SLe^a, instead of its normal counterpart disialyl Lewis a (di-SLe^a). SLe^a has been reported to play a crucial role in cancer invasion/metastasis by acting as ligand for endothelial cell E-selectin, which is responsible for cell adhesion [115–118].

It is worth noting that majority of these glycobiomarkers were discovered by generating tumour-specific monoclonal antibodies (mAbs). In addition to aid in the discovery of additional carbohydrate-based biomarkers, these tumour-specific mAbs have great potential in treating neoplastic disease.

4.4. Antibody-based immunotherapy of cancer

Specific recognition and elimination of malignant cells by antibodies were proposed over a century ago [119]. The development of antibody-based therapies for cancer has been the focus of considerable interest for decades. Several criteria have been described for the selection of antitumour mAbs: (1) the mAb binds to cell surface tumour antigen, (2) the mAb binds to tumour antigen at high affinity, (3) the mAb recognises tumour antigen that is overexpressed on tumours but has limited expression on normal tissues, (4) the mAb has potent immune-mediated and non-immune-mediated cytotoxicity effects, (5) the mAb directly kills tumour cells and/or (6) the mAb internalises into target cells so it can delivery toxic payloads. To date, many therapeutic monoclonal antibodies (mAbs) have been developed, mostly against protein antigens, and have proven useful in cancer therapy.

4.5. Anti-glycan monoclonal antibody against cancer

Anti-glycan mAbs have also found usage in clinical applications. Dinutuximab is a chimeric mAb directed against GD2 on neuroblastoma and induced cell lysis via Antibody dependent cellular cytotoxicity (ADCC) and Complement dependent cytotoxicity (CDC) (<http://www.fda.gov/>). It was approved by FDA in 2015, for use in high-risk neuroblastoma paediatric patients, in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-2 (IL-2) and 13-cis-retinoic acid (RA). GD2 is a disialoganglioside overexpressed on neuroblastoma and melanoma [79, 120] with limited expression on normal neurons in the cerebellum, skin melanocytes and peripheral nerves [121], making it well suited as target for cancer therapy. The FDA approval was based on the findings from a phase III trial, focused on 226 children with high-risk neuroblastoma who had responded to initial treatment [122]. Patients were enrolled to receive either Dinutuximab associated with IL-12, GM-CSF and RA or RA alone, after having responded to the first-line treatment. Three years after treatment assignment, patients received Dinutuximab combination showed superior rates of event-free survival and overall survival when compared to standard therapy.

BR96 is an anti-Le^y mAb. It was shown to induce direct tumour cell death to Le^y-positive tumour cell lines in addition to ADCC or CDC [123]. In a phase I trial, BR96-doxorubicin immunoconjugates showed limited clinical antitumour activity with patients experiencing a clinically significant hypersensitivity reaction to the mouse man [124]. BR96 was conjugated to doxorubicin and docetaxel (also known as SGN-15), and 62 advanced non-small-cell lung cancer patients were treated in a randomised Phase II trial. An increased in survival was

reported for patients receiving SGN-15 when compared with patients receiving doxorubicin and docetaxel alone [125]. hu3S193 is a humanised anti-Le^x mAb. It contains only 3–5% of murine residues in the antibody variable domain, conferring a low risk of hypersensitivity responses. In a MCF-7 xenograft preventive model, hu3S193 managed to significantly slow tumour growth compared with placebo and isotype-matched control IgG1 antibody [126]. In a phase I trial in 15 cancer patients (six breast, eight colorectal and one non-small-cell lung cancers), hu3S193 showed only minimal toxicity. The biodistribution of indium 111 radio-labeled hu3S193 ((111)In-hu3S193) showed no evidence of normal tissue uptake, but (111)In-hu3S193 uptake was seen in cutaneous, lymph node and hepatic metastases [127].

KM231 is a murine mAb recognising SLe^a. KM231 was observed to react with many human gastrointestinal cancer tissues and could detect shed antigen in the sera of cancer patients. Shitara et al. made KM231-ricin A chain immunotoxin to evaluate the tumoricidal effect of KM231 on ascites and subcutaneous xenograft tumours growing in nude mice. KM231 significantly inhibited the growth of established subcutaneous tumours. This result suggested that it was an effective tumoricidal drug when it was conjugated to cytotoxic reagents [128]. 5B1 (IgG1) and 7E3 (IgM) are other anti-SLe^a mAbs generated by immunising mice with SLe^a-KLH vaccine. Both mAbs are very potent in inducing CDC. Moreover, 5B1 is also highly active in inducing ADCC [129].

NCCT-ST-421 (IgG3) is a murine mAb raised by immunising mice with human gastric cancer xenograft (ST-4). The mAb recognises dimeric Le^a epitope and cross reacts with simple Le^a and extended Le^a epitopes. NCCT-ST-421 induced ADCC and CDC to antigen-positive cells. It was shown to induce direct cell death as well through apoptotic mechanism. Although NCCT-ST-421 showed promising antitumour responses, no further details regarding clinical studies were described.

4.6. Direct killing anti-glycan monoclonal antibody

Oncosis is a progressive cell death process initially involving the impairment of ionic pumps of the cell membrane accompanied by cellular and organelle swelling. Subsequently, a gradually increase in membrane permeability due to an increasing cytosolic calcium concentration as well as rearrangement of cytoskeletal proteins results in pore formation in the cell membrane [130, 131]. It has been well accepted that tumours are able to manipulate the tumour microenvironment by releasing cytokines and other soluble factors, which create an immunosuppressive environment [132]. Release of cellular content into immunosuppressive tumour microenvironment via mAb-induced pore formation during oncosis may help in evoking immune responses via the release of danger-associated molecular patterns ('DAMPs'; [133]).

FG88 (FG88.2 and FG88.7) are internalising murine IgG3 mAbs recognise Le^{a-c-x} glycans (**Figure 6**) overexpressed on a wide range of tumour cells and tissues at subnanomolar potency [134]. The FG88.2 mAb showed excellent tumour cell surface antigen binding and good levels of binding to a large percentage of tumours with low level binding to a limited number of normal tissues by immunohistochemistry. The significant association of strong FG88.2 binding with poor outcome in the colorectal sample cohort, independent of stage and vascular invasion, suggests that FG88.2 mAb has great potential targeting the most aggressive

Glycan structure	Name
Lewis a
Di-Lewis a
Lewis a Lewis x
Lewis c Lewis x
Lea-containing glycan

Figure 6. Details of glycan binding by FG88 mAbs.

colorectal cancers. FG88 mAbs induce potent ADCC, CDC and direct killing of tumour cells via oncosis. In the in vivo xenograft study, FG88 mAbs eradicated both primary and meta-static tumours. By releasing tumour cellular contents via mAb-induced pores, the FG88 mAbs might also be able to reverse the immunosuppressive tumour microenvironment leading to effective presentation of multiple epitopes from the lysed tumour cells and amplifying the antitumour immune response.

Several other anti-glycan mAbs can also induce similar direct tumour killing to FG88. MAb 84, when bound to human embryonic stem cells, induced cytoskeletal protein (α -actinin, paxillin and talin) degradation, which in turned increased the mobility of the plasma membrane, resulting in the clustering of antigens on the cell surface. Following antigen clustering, formation of pores through the plasma membrane resulted in oncosis [135]. N-glycolylneuraminic acid (NeuGc) is a sialic acid variant of N-acetylneuraminic acid (NeuAc). Humans cannot synthesise NeuGc because they lack the enzyme cytidine mono-phospho-N-acetylneuraminic acid hydroxylase (CMAH), responsible for its biosynthesis.

However, recent evidence suggests that NeuGc can be incorporated into human glycan from dietary sources. More interestingly, although not fully understood, human tumours actively incorporate NeuGc at a much higher rate than normal primary cells. As such, it is highly expressed in several human cancer cells [136], making it an appealing target for immunotherapy. It is noteworthy that natural circulating antibodies to NeuGc can be detected in normal human serum and that these antibodies have been shown to induce complement-dependent cell lysis [137]. Anti-NGcGM3 14 F7 mAb induced rapid cell death which was accompanied by cellular swelling, membrane lesion formation and cytoskeleton activation and cell aggregation. Moreover, no evidences of DNA fragmentation, chromatin modification or caspase activation were found. But 14 F7-treated cells showed large lesions at the plasma membrane, much bigger pores created by complement, perforin or bacterial toxins, suggesting an oncosis-like phenomenon [138]. Currently, the NeuGc-ganglioside anti-idiotypic mAb, Racotumomab (1E10), is under development. By molecular mimicry, a selected anti-idiotypic mAb will behave like the original antigen. In a phase III randomised trial in non-small-cell lung cancer, Racotumomab conferred a significant survival advantage [139] and induced anti-NeuGc antibodies capable of killing tumour cells by a mechanism similar to oncosis, validating the approach and highlighting the beneficial value of antibodies mediating oncosis for therapeutic purposes [140].

5. Conclusion

Glycoconjugates are major components of cells. They are involved in defining and modulating multiple key physiological processes in normal tissues. Aberrant glycosylation in cancer lead to the modification of glycosylations, resulted in the generation of TACAs, which drive several biological processes in cancer. Investigation of the molecular basis underlying these glycan modifications will aid in the understanding of cancer immunology as well as the development of anti-glycan therapeutic mAbs. Furthermore, rapid advances in glycomics and glycoproteomics will have a major impact on the unravelling of novel targets for cancer treatment.

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