Glycosylation in cancer: Selected roles in tumour progression, immune modulation and metastasis

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ABSTRACT

Tumour metastasis is the main cause of cancer related deaths. Metastasis is an intricate multi-step process that requires the acquisition of several cancer cell features, including the modulation of tumour cell migration, adhesion, invasion, and immune evasion. Changes in the cellular glycosylation are associated with malignant transformation of cancer cells, tumour progression and ultimately, metastasis formation. Glycans have major impact on cellular signalling and on the regulation of tumour cell-cell adhesion and cell-matrix interaction. Glycans drive the interplay between the cancer cells and the tumour microenvironment. In this review, we summarize the roles of glycan alterations in tumour progression, such as acquisition of oncogenic features due to modulation of receptor tyrosine kinases, proteoglycans, cadherins and integrins. We also highlight the importance of key glycan binding proteins such as selectins, siglecs and galectins, which are pivotal in the modulation of immune response. An overview on glycans as cancer biomarkers is also presented.

Keywords: Glycosylation in Cancer; Biomarkers; Receptor Tyrosine Kinase; Cadherins; Integrins; Proteoglycans, Selectins; Galectins; Siglecs; Metastasis.

Abbreviations: AFP-L3, α1,6-fucosylated α-fetoprotein; BPH, benign prostatic hyperplasia; CEA, carcinoembryonic antigen; CS, chondroitin sulphate; CRD, carbohydrate recognition domains; CTC, circulating tumour cell; ECM, extracellular matrix; EMT, epithelial to mesenchymal transition; EV, extracellular vesicle; FAK, focal adhesion kinase; GAG, glycosaminoglycan; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; GnT, N-acetylglucosaminyltransferase; GPC, glypican; HS, heparin sulphate; HSPG, heparan sulphate proteoglycan; ITGB1, integrin β-1; NCAM,

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neuronal cell adhesion molecule; NK, natural killer; PG, proteoglycan; PSA, prostate specific antigen; RTK, receptor tyrosine kinase; SDC, syndecan; SLea, sialyl Lewis A; SLex, Sialyl Lewis X; STn, sialyl Tn; TGM, tissue transglutaminase; TMTC, transmembrane and tetratricopeptide repeat-containing protein.

1. GLYCANS IN CANCER

Glycosylation is one of the most frequent protein and lipid modification, playing an essential role in the normal development and physiology of cells [1]. Through the tight regulation and dynamic action of a variety of enzymes, namely glycosyltransferases and glycosidases, glycosylation is able to produce a wide range of glycan structures, which can exist free or conjugated to proteins or lipids [1–3]. In this review, we address the role of altered protein N-glycosylation, O-glycosylation, glycosaminoglycans (GAGs) and glycolipids, and the impact on their carrying glycoconjugates as well as the function of key glycan binding proteins in cancer metastasis.

In glycoproteins, the linkage of the glycan chains to their polypeptide backbone typically occurs via nitrogen of asparagine (N-glycans) or oxygen of serine or threonine (O-glycans). N-glycans are oligosaccharides covalently linked to an asparagine at the peptide sequence Asn-X-Ser/Thr (where X represents any amino acid except proline). N-glycans share a common pentasacharide core region and can be divided in three main types: high-mannose, hybrid or complex. O-glycans are often densely clustered on serine and threonine rich protein domains, as it is the case in mucins. Glycosaminoglycans (GAGs) are large chains of linear repeating disaccharide units which can be either linked to proteoglycans or appear as free saccharides, such as hyaluronan [1–4].

Most glycans and glycoconjugates can be found in the outer surface of the cellular membrane, creating a dense coat of sugars surrounding the cells, the glycocalix. Given their position, it is not surprising that glycans play a major role in recognition and interaction processes between cells and their extracellular environment, mediating cell adhesion, cell-matrix interactions, cellular signalling, as well as host-pathogen interactions [1,3,5].

Altered expression of glycans and glycoconjugates has been associated with numerous pathologies, including congenital disorders [6], immunodeficiencies and cancer [3,4,7]. Several mechanisms have been described underlying changes in both N- and O-glycan structures. Aberrant glycosylation is the result of alterations in the cellular and molecular machinery. These include altered glyco-related gene expression, glyco-enzyme localization in the Golgi apparatus and availability of acceptor and donor substrates. Glycan alterations lead to impaired cell-cell adhesion, activation of oncogenic signalling pathways and induction of pro-metastatic phenotypes [3,8,9]. A metastatic tumour cell has to overcome cell-cell adhesion in order to detach from the primary tumour cells, migrate and invade the surrounding tissue, enter blood or lymphatic vessels, disseminate throughout the body and finally endure extravasation to metastasize into different organs. Cadherins are one of the major families of cell adhesion molecules known to be regulated by glycan structures with impact in cancer progression [9–11]. Similarly, integrins glycosylation changes are crucial to assist cancer cell invasion [3,9]. In

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addition, specific glycan alterations have also been identified as a key players in the oncogenic activation of receptor tyrosine kinases (RTKs) [12–14].

Malignant tumour cells are known to express different subsets of glycan epitopes, including truncated simple O-glycans, changes in N-glycan branching, increased sialylation, fucosylation, and altered GAG [2,3]. Aberrant terminal sialylated glycoforms frequently modulate the interaction with carbohydrate binding proteins. Selectins and their ligands, for instance, mediate a highly regulated system of cell adhesion between leukocytes and vascular endothelial cells, an important process during inflammation [5,15], which is mimicked by the tumour cells in the metastization process. Moreover, glycans are also involved in the modulation of the immune response with glycan binding proteins, such as siglecs and galectins, regulating key immune cell functions [3,5,16,17].

Furthermore, the glycosylation alterations present in malignant tumour cells and their implication in different steps of tumour progression have major applications as biomarkers for patient diagnosis and prognosis (Figure 1) [2,3,7,18,19].

In this review, we will discuss different mechanisms through which glycosylation has been shown to regulate cancer progression steps leading to metastasis.

2. GLYCOSYLATION MODULATION OF RECEPTOR TYROSINE KINASE ACTIVITY IN CANCER

Receptor tyrosine kinases (RTKs) are glycosylated transmembrane cell surface receptors that regulate cellular signalling processes, often in response to paracrine or endocrine stimuli. These include cell division, differentiation, migration and angiogenesis, which are key processes in malignant transformation and cancer progression. As a result, RTKs are among the most consistently altered gene/protein class in cancer, often leading to the constitutive activation of the receptor and thus driving tumour cell invasion and metastasis [20].

In the past, the hyperactivation of RTKs was mainly attributed to the overexpression or mutational alterations of these receptors [20]. However, it became evident in recent years that alterations in glycosylation can cause the aberrant activation of RTKs (Figure 2A). These changes cannot be detected on gene, transcript or protein levels of the receptor and have been therefore often overlooked.

Alterations in glycosylation leading to increased RTK activation can stem from two main reasons: transactivating glycoconjugates or altered glycosylation of the RTK itself. Famous examples of transactivating glycoconjugates are GAGs, which can tether RTK ligands and thus, enhance receptor activation [21–23]. Also gangliosides (sialylated glycosphingolipids) have been described as important modulators of RTK activation and signal transduction. For instance, RTKs are located in glycolipid-enriched microdomains which can promote or impede their activation [24,25]. Receptors such as EGFR, FGFR, MET and IGFR have been described to be regulated by gangliosides [24–26]. In this regard,

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monosialogangliosides appear to be negative regulators and disialogangliosides positive regulators of RTK activation [26].

Regarding aberrant glycosylation of RTKs, it has been described that RTKs that stimulate cell proliferation when activated (e.g. EGFR, FGFR, IGFR and PDGFR) have evolutionary more N-glycan sites (8–16 sites) per 100 amino acids, and longer extracellular domains, as compared to growth-arrest receptors involved in organogenesis and differentiation (e.g. TGFβR1 and TGFβR2) [12]. It has been demonstrated that N-glycosylation can function as a metabolic master regulator of cell proliferation and arrest through modulation of RTK glycosylation [12]. In addition, altered glycosylation, including changes in sialylation and fucosylation of EGFR affects its dimerization and its activation in lung cancer cells [27]. Furthermore, sialylation of EGFR has been shown to regulate the receptor activity and chemosensitivity to gefitinib in colon cancer cells [28].

The importance of N-glycosylation for RTK function is further highlighted by studies that blocked N-glycan formation in cancer cells [13,29,30]. These studies show that RTKs are particularly sensitive to the impediment of N-glycosylation, either by inhibitors or gene silencing, leading to the abrogation of RTK induced signal pathways and cell growth arrest of RTK dependent cancer cells [13,30,31]. The degree of N-glycan branching, promoted by MGAT5 and inhibited by MGAT3, dictates the response to the RTK ligands EGF, FGF, IGF and PDGF in carcinoma cells [32–37]. The proposed mechanism, particularly well-studied for EGFR, is that N-glycan branching promotes the galectin mediated retention of the receptor at the cell surface and thus, facilitates the RTK ligand dependent stimulation [12,33,35].

In addition, the sialylation status of N-glycans has been shown to be particularly important for RTK membrane retention and activation. In this regard, sialic acids linked α2-6 to N-glycans have been demonstrated to interfere with galectin-1 binding and this mechanism is of importance for VEGF signalling of endothelial cells, as the reduction of α2-6 sialic acids enables VEGF independent tumour angiogenesis in cancer [38].

In gastric carcinoma, the increase of α2-3 sialylation, commonly observed by the increased formation of sialyl Lewis epitopes, leads to a more invasive phenotype through the hyperactivation of the RTKs MET and RON [14,39]. Interestingly, the use of anti-sialyl Lewis A antibody CA19-9 was shown to be sufficient to abolish the ERBB2 hyperactivation in gastric cancer cells [29].

3. GLYCOSYLATION OF ADHESION MOLECULES IN CANCER

3.1. Glycans and cell-cell adhesion

The process of carcinogenesis, tumour progression and the consequent formation of metastasis is intimately related to the loss of cell-cell adhesion, followed by the acquisition of migratory capacity and invasion of surrounding tissues [40], a process that frequently involves the loss of epithelial characteristics and gain of mesenchymal features, known as epithelial to mesenchymal transition

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(EMT). Alterations on the cellular profile of glycans have been described to regulate the migratory and invasive capacity of cancer cells contributing to metastasis [3].

E-cadherin is a transmembrane glycoprotein described as the main cell-cell adhesion molecule in epithelial tissues, with key roles in the EMT process [41–43]. The dysregulation of the E-cadherin function in cancer has been described to occur through different mechanisms including alterations in N-glycosylation (Figure 2B) [10,42,44]. Human E-cadherin exhibits four potential N-glycosylation sites [45] and alterations on the expression profiles of E-cadherin-linked N-glycans are associated with malignant and invasive phenotypes, as well as poor survival in cancer patients [46,47]. The prevention of the abnormal N-glycosylation of E-cadherin at Asn-554 was associated with a protective effect [48,49]. N-glycosylation is also crucial for the stability of N-cadherin in glioma cells and plays a role abrogating cell-cell adhesion and promoting tumour cell migration [50].

Tumour cells often exhibit an increased activity of N-acetylglucosaminyltransferase V (GnT-V), which is encoded by the human $MGAT5$ gene [47,51,52]. The addition of GnT-V-mediated β1,6GlcNAc-branched N-glycans on E-cadherin is able to induce alterations on its cellular localization, from the cell membrane to the cytoplasm, impairing its biological functions [47,53] and favouring a more invasive and metastatic phenotype [18,54]. On the contrary, the modification of E-cadherin with bisecting N-acetylglucosamine (GlcNAc) N-glycans catalyzed by N-acetylglucosaminyltransferase III (GnT-III) was demonstrated to impose opposite effects on E-cadherin functions through competition with the GnT-V enzyme [53,55]. This bisecting N-linked glycans on E-cadherin was described to increase the stability of adherens junctions, leading to tumour suppression [11,47,53]. Moreover, a study with breast cancer cells lacking E-cadherin expression showed that the addition of exogenous E-cadherin culminated on the inhibition of insulin receptor signalling pathway. Besides, the stimulation of these cancer cells with insulin or insulin-like growth factor 1 led to a decrease of bisecting GlcNAc N-glycans on E-cadherin together with its cellular mislocalization and increased tumour cell invasion [36,37].

There is yet another level of regulation of E-cadherin functionality by O-mannosylation, a process that is canonically catalyzed by the O-mannosyltransferases 1 and 2 [56], with new evidences for the contribution of a homologous family of putative O-Man glycosyltransferases encoded by the four transmembrane and tetratricopeptide repeat-containing protein genes (TMTC1–4) [57]. O-mannosylation is generally characterized by the attachment of mannose residues to serine or threonine amino acids [58–60]. Functional E-cadherin displays high levels of O-mannosylated glycans whereas the non-functional E-cadherin exhibits an altered pattern of protein glycosylation, namely increased β1,6GlcNAc-branched N-glycans and decreased O-mannosylation glycans that contributes to cancer progression [61].

Sialylation is also crucial for the regulation of cell adhesion process. Increased expression of sialylated glycans contributes to cell spread from the tumour mass, possibly by electrostatic repulsion, with consequent surrounding tissues invasion [62,63]. In breast cancer cells, the expression of ST6GAL1 led

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to a reduction of cell-cell adhesion and an enhanced invasion capacity [64]. In gastric cancer, aberrant expression of sialyl-Tn (STn) was described to regulate the tumour aggressive phenotype through a negative impact on cell-cell adhesion with consequent tumour cell migration and invasion [65,66]. In accordance with this, functional alterations on the C1GALT1-specific chaperone 1 (C1GALT1C1), caused by somatic mutations and hypermethylation of the C1GALT1C1 gene, promoted the overexpression of premature truncated glycans such as STn, leading to the impairment of cell adhesion, associated with invasion and metastasis [67]. Furthermore, silencing of ST6GALNAC1, enzyme responsible for the synthesis of STn [66], culminates on a decrease of metastatic capacity of gastric cancer cells [68]. At clinical level, cancer patients that present increased sialylation are frequently associated with venous invasion and poor survival rates [66,69,70]. In addition, polysialic acid, commonly expressed in NCAM (neuronal cell adhesion molecule), is implicated in tumour development and metastasis [71,72].

3.2. Glycans and cell-matrix interactions

The extracellular matrix (ECM) is the non-cellular portion of a tissue comprised of a complex network of collagens, glycoproteins, proteoglycans and GAGs. The integrin family encompasses the major surface receptors involved in the adhesion of cells to the ECM elements. Changes in dynamics of cell-ECM interactions, including those orchestrated by glycans, are crucial for the acquisition of migratory and invasive behaviour during carcinoma progression [73].

Particularly N-linked glycans modulate integrin function regulating the migration capacity of tumour cells [74]. Similar to E-cadherin, the branched and bisected N-glycans affect integrin functions in an opposite manner. In gastric cancer cells, the overexpression of MGAT5 significantly enhanced the expression of β1,6GlcNAc-branched glycans on integrin α3β1 leading to increased tumour cell migration on the laminin 5 substrate; whereas the overexpression of GnT-III led to the opposite effect [75].

The expression of branched versus bisected N-glycans on the integrins also modulates its signalling activity [76]. The overexpression of GnT-III in HeLa S3 cells led not only to a suppression of the integrin α5β1-mediated cell migration but also to decreased levels of focal adhesion kinase (FAK) phosphorylation [77]. Moreover, specific N-glycosylation sites on integrins have been shown to regulate different cellular functions, such as RTK activation [78,79]. Interestingly, it has been demonstrated that the levels of GnT-V are controlled by the RAS oncogene [80]. Since integrin-linked N-glycans modulate the signalling activity, which in turn affects the branched/bisected N-glycan balance, we might speculate about the possible existence of a reciprocal regulatory mechanism.

Fucosylation and sialylation are also implicated in the modulation of integrin functions. In HepG2 liver cancer cells, the inhibition of fucosylation suppressed migration as well as integrin β1-related intracellular signalling through FAK [81]. Moreover, the overexpression of ST3GAL3 in pancreatic adenocarcinoma cells promoted high levels of Sialyl Lewis X (SLex) and lower levels of α2,6-sialic acid

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content on the α2β1 integrin-linked N-glycans, leading to enhanced FAK phosphorylation and increased invasive phenotype [82]. Interestingly, increased α2,6-sialylation promoted integrin α5β1-dependent hepatocellular carcinoma cell adhesion in a mouse cell line [83].

4. PROTEOGLYCANS AND GLYCOSAMINOGLYCANS INVOLVED IN CANCER

GAGs comprise a major class of oligosaccharide structures in the ECM [84–87]. GAGs are long unbranched polysaccharides of high molecular weight. They consist of repeating disaccharide units, composed of either GlcNAc or N-acetylgalactosamine (GalNAc) and either uronic acid or galactose. GAGs are ubiquitous in mammals and have an important role in the regulation of cellular behaviour and function by interacting with other ECM components like plasma proteins, growth factors, cytokines or amino acids. GAGs are divided in five major categories: hyaluronan, chondroitin sulphate (CS), dermatan sulphate, heparin and heparan sulphate (HS) and keratan sulphate [85–88]. Proteoglycans are a family of structural and functional biomolecules ubiquitously present in ECM and cell surface playing key roles in ECM structural organization and cell signalling, contributing to the control of numerous physiological and pathological processes [89]. As multifunctional molecules, proteoglycans participate in various cell functions during morphogenesis, wound healing, inflammation and tumorigenesis. Proteoglycans consist of a core protein onto which one or more GAGs are covalently attached [90,91]. According to location, proteoglycans are classified in intracellular, cell surface and extracellular proteoglycans [91].

4.1. Intracellular Proteoglycans

Serglycin is the only true intracellular proteoglycan discovered so far [92]. However, serglycin can also be found as an extracellular proteoglycan. The intracellular form mediates granulopoiesis and is involved in the retention and secretion of molecules, such as proteases, cytokines and chemokines by inflammatory cells. The extracellular serglycin plays an important role in cell-cell interactions and contributes to extravasation, colonization and growth of metastatic cells [93]. Increased expression of serglycin is associated with poor prognosis in mammary and nasopharyngeal carcinomas [94,95]. Genetic ablation of serglycin prevents lung metastasis in breast cancer mouse models [94]. The increased expression of this proteoglycan in nasopharyngeal carcinoma cells led to enhanced cancer cell motility, invasion and metastasis [95].

4.2. Cell Surface Proteoglycans

Two major families of membrane-associated proteoglycans are syndecans and glypicans. In mammals, syndecan (SDC) is a family of four distinct members (SDC1-4) which are predominantly modified with HS chains and thus belong to the heparan sulphate proteoglycans (HSPGs) [89]. The HSPGs can signal

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and regulate important cell processes, such as adhesion, migration, proliferation and differentiation. HSPGs, due to its ability to fine-tune molecular interactions, are highly relevant to tumour progression [96]. In addition, it has become apparent that the enzymes that regulate glycan modification of HSPGs are also powerful regulators of tumour behaviour [97]. The HSPGs act cooperatively with integrins to mediate adhesion of cells to the ECM and focal adhesions formation. Disruption of cancer cell focal adhesions dictates cell migration and invasion capacity. The key role of HSPGs in metastasis formation was demonstrated by altered expression of HSPGs in breast cancer [98]. In addition, many preclinical studies and clinical trials support the investigation of anti-metastasis agents targeting HSPGs [99].

The loss of expression of SDC1 is a characteristic feature of human hepatocellular carcinomas with high metastatic potential [100]. On the other hand, SDC1 expression was associated with development, differentiation and clinical stage in colorectal cancer but not in metastasis [101]. The overexpression of SDC1 may modulate the biosynthesis and sulfation of HS affecting the expression of other proteoglycans. At transcript level, the overexpression of SDC1 has been proven to affect genes involved in growth regulation and EMT of tumour cells [102].

SDC2 overexpression enhanced the SDC1-mediated malignancy of mesenchymal tumour cells [103]. The upregulation of SDC2 is associated with development of colon cancer, and affects the cancer activity regulation in invasive capacity in lung and colon cancer cells [104,105]. The reduction of SDC2 levels induces apoptosis and may abolish growth and metastasis in breast tumour [106].

In turn, SDC3 is frequently expressed in pancreatic cancer and contributes to a poor prognosis [107].

The loss of expression of SDC4, which is ubiquitously expressed, is related to increased metastatic potential, vascular/lymphatic invasion and disease stage in non-seminomatous germ cell tumours [108]. SDC4 overexpression contributed to the development and metastasis formation of renal cell carcinoma [109] as well as to a more aggressive clinical behaviour of osteosarcomas [110].

The glypican (GPC) family consists of six members of HSPGs anchored to the external surface of the cell membrane via a GPI (glycosylphosphatidylinositol) [111,112]. In recent years the importance of GPCs in the tumorigenic process has become evident. In this regards, GPC2 and GPC3 are associated with poor prognosis in neuroblastomas and hepatocellular carcinomas, respectively [113,114], whereas GPC5 has been reported as a tumour suppressor in prostate, lung and breast cancers [115–117].

Neuron glia antigen-2/ Chondroitin Sulphate Proteoglycan 4 (NG2/CSPG4) is an integral membrane proteoglycan located on the surface of many cell types. It is a prominent component of activated pericytes and plays important functions in pericyte recruitment and vascular morphogenesis [118–120]. NG2/CSPG4 upregulation is a poor prognostic marker for hepatocellular carcinoma, head and neck patients [121,122].

4.3. Extracellular Proteoglycans

Extracellular proteoglycans, such as versican and perlecan, are important components of the ECM. Versican is a large CS proteoglycan and has the ability to modulate tumour progression in different

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carcinomas including breast, gastric, renal, ovarian clear cell carcinoma and osteosarcoma [123–127]. Using mouse models that spontaneously develop breast cancer, it was demonstrated that myeloid progenitor cells in the lung pre-metastatic niche express versican and the knockdown of this ECM component in the bone marrow impaired lung metastasis [128].

Perlecan is a HSPG found at tissue borders and it is described to be involved in dysplastic changes of epithelial cells. The accumulation of perlecan within the cell and the intercellular space serves as a reservoir for various growth factors helping in tumour progression, angiogenesis and metastasis in prostate tumour, laryngeal and oral squamous cell carcinomas [129–132].

Alterations affecting the amount or composition of glycans present in the ECM can trigger phenotypic changes favouring the development of tumours. The excessive hyaluronan production by murine breast cancer cells led to the expansion of cancer stem cells and the development of aggressive carcinomas [133]. On the other hand, the enhanced degradation of the ECM by overproduction of hyaluronidase 1 in human prostate carcinoma cells led to an increase of motility and proliferation, allowing also the invasion of the basement membrane and the clearing of the ECM on the metastatic site [134,135]. ECM changes are also implicated in the formation of pre-metastatic niches [136].

5. SELECTINS IN METASTASIS

A mechanism by which aberrant glycosylation enhances metastatic properties in cancer cells is by the expression of selectin ligands at the cell surface, such as SLex. Selectins are a family of multifunctional adhesion glycoproteins that bind to carbohydrates in a calcium-dependent manner. They are type I membrane proteins that consist of an N-terminal lectin domain, an EGF-like module, followed by 2-9 consensus repeats, a transmembrane region and a short cytoplasmic tail [137,138]. There are three members of the selectin family: E-, L- and P-selectin that differ in the number of the consensus repeats, their cell type dependent expression patterns and the biological functions. E-selectin is expressed exclusively on the activated endothelium, L-selectin is predominantly expressed on leucocytes and P-selectin on activated platelets and endothelial cells [139]. Selectins are key players in physiological and disease-related processes such as inflammation, by mediating the adhesion of leucocytes and platelets with the endothelium in the bloodstream. Nevertheless, selectins and their ligands are not only involved in processes such as leukocyte homing but also cancer [140].

During inflammation, and upon cytokine stimuli, E-selectin is expressed on endothelial cells promoting the rolling, arrest and transmigration of leucocytes. This process is mediated by the interaction of selectins with SLex and SLea containing glycoproteins and glycolipids on leucocytes. A similar process has been claimed in cancer cells. Interactions of cancer cells with selectins have been well documented in various cancer types and associated with an enhanced metastatic capacity [141–143]. In addition, P-selectin cancer cell interaction is described to promote the formation of platelet-cancer cell micro-emboli being associated to tumour metastasis [144]. Besides, the constitutive expression of E-selectin

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on the microvascular bone has been reported to correlate with the metastatic bone tropism of breast cancer cells overexpressing SLex [145].

Cancer cell selectin interaction requires the expression of SLex and sulfation of the glycan structure, as well as its structural isomer SLea. The biosynthesis of these cancer associated antigens depends on the combined action of specific fucosyltransferases, galactosyltranferases and α2-3-sialyltransferases, being the α2-3-sialyltransferases ST_3GAL_3, ST_3GAL_4 and ST_3GAL_6 the key enzymes in the biosynthesis of E- and P-selectin ligands [146]. Aberrant expression of both sialyltranferases and their cancer associated antigens SLex and SLea are features of cancer cell transformation [147] and this deregulated expression has been associated to metastatic tumours and poor patient outcome [3].

As mentioned above, the activation of E-selectin on the endothelium is cytokine-dependent and known to be induced through the Ras/Raf/mitogen-activated protein kinase pathway [148]. In an analogous way, primary tumours can promote an inflammatory activation of the endothelium in distant organs facilitating metastatic spread [149,150]. For instance, colon tumour cells upon entry to the hepatic circulation can induce a cytokine cascade effect, resulting in the expression of E-selectin on the sinusoidal endothelium [150,151]. The expression of E-selectin on activated hepatic sinusoidal endothelium can then interact with carbohydrate ligands present on colorectal cancer cell molecules, including CD_{44} and CEA, mediating liver metastasis [152–155]. Furthermore, direct evidences have pointed out the role of hypoxia in promoting the expression of enzymes responsible for the synthesis of selectin ligands. It has been demonstrated that cells grown in hypoxic conditions have a remarkable increased expression of ST_3GAL_6 and concomitant increased expression of selectin ligands [156]. In addition, increased expression of ST_3GAL family members that led to the expression of SLea and SLex was also described to mediate selectin binding during EMT [157].

Altogether, these observations support the role of cancer cell selectin interactions and the formation of micro-emboli, due to alternated glycosylation during tumorigenesis, in enabling cancer cell adhesion to endothelium, protection from immune elimination and formation of metastatic foci.

6. Tumour Immunity: the role of Siglecs and Galectins

Tumour cell glycans, displaying specific epitopes, enable cell binding and interaction with microenvironment through glycan-binding receptors such as lectins [158]. This lectin-glycan recognition modulates the immune response through binding to immune cell receptors.

6.1. Siglecs

Siglecs, sialic-acid-binding Ig-like lectins present on virtually all hematopoietic cells, are divided into two major subgroups based on their sequence similarity and conservation among mammalian species. The first group comprises siglec-1, -2, -4 and -15 in humans, which have orthologs in all mammalians and share about 25-30% sequence identity. The second group comprises nine siglec-3 (CD_{33})-related siglecs, which share >50% sequence identity and appear to be evolving rapidly [159]. This rapid

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evolution was termed "Red Queen effect" by the need to overcome the rapidly evolving sialome of the host [160]. Most siglecs bind sialic acid ligands either in cis or trans and have one or more tyrosine-based signalling motifs in their cytoplasmatic tails or associate with membrane adaptor proteins containing cytosolic tyrosine motifs [1]. In recent years, several experimental models have provided evidence that siglecs are implicated in immune evasion and cancer progression. Analyses of hypersialylated ligands on tumour cells and secreted proteins within the tumour microenvironment have identified mucins and also secreted N-glycosylated glycoproteins to be high-affinity ligands for siglecs [161]. Siglec-1 has been reported to bind MUC1 [162], which is aberrantly overexpressed in a wide range of tumours and associated with poor prognosis [163]. Similarly, siglec-9, present on immune cells, also binds prominently to MUC1 [164] and dependent binding to MUC1-sialyl T to promote the secretion of factors associated with tumour progression [165]. Siglec-9 can also be a receptor for MUC16, that acts as an adhesion molecule and facilitates peritoneal metastasis of ovarian tumours, thus likely mediating inhibition of anti-tumour immune responses [166]. The levels of siglec-7 and siglec-9 ligands seem to be greatly elevated on different human cancer cells, decreasing their susceptibility to Natural Killer (NK) cell killing [167,168]. Furthermore, downregulation of NK cell cytotoxicity in an α2,6-linked disialic ganglioside-siglec-7-dependent manner was shown to create favourable circumstances for survival and metastasis of renal cell carcinoma cells [169], evidencing the role of these siglecs as inhibitory receptors.

The highly restricted and differential expression of siglecs in the cells of the immune system is prompting them as targets for development of immunotherapeutics. Siglec-2 has the highest conserved specificity, binding primarily α2,6-linked sialylated ligands. In a metastatic model of lung cancer, an anti-siglec-2 antibody prevented the development of lung metastasis and improved survival [170]. In addition to antibody-based, other strategies such as nanoparticles decorated with glycan ligands are being developed [17].

6.2. Galectins

Galectins are soluble proteins that through their carbohydrate recognition domains (CRDs) recognize β-galactosides in the extracellular milieu by interacting with numerous glycosylated receptors or, intracellularly, by controlling signalling pathways through protein-glycan or protein-protein interactions. The galectin family consists of 15 members, classified into three groups based on structural differences: prototype galectins (galectin-1, -2, -5, -7, -10, -11, -13, -14 and -15) with one CRD, tandem repeat-type galectins (galectin-4, -6, -8, -9 and -12) with two CRDs and the unique galectin-3, with one CRD connected to a non-lectin N-terminal region responsible for oligomerization [171]. They are increasingly recognized as important players in metastasis due to their effects on cancer cell–matrix, cancer cell–cell and cancer-endothelial adhesion [172]. Profiling galectin expression in endothelial cells revealed abundant expression of galectin-1, -3, -8 and -9 and lower expression of galectin-2, -4 and -12, evoking a therapeutic potential independent of tumour type [173]. Many

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galectins exhibit strong binding to β1,6-branched glycans and diminished binding to β-galactosides capped with α2,6-sialic acid, as a result of increased expression of ST6GAL1 during carcinogenesis [174,175], which may protect cancer cells from galectin-mediated apoptosis and promote tumour cell survival. Galectin-3-N-glycan complexes favour anergy of tumour-specific cytotoxic T cells [176] and interactions between galectin-3 and complex N-glycans on CTLA-4 may delay the endocytosis of this immune checkpoint receptor and prolong delivery of arrest signals upon T cell activation [12]. Galectin-3 interaction with cancer-associated MUC1 via the T-antigen, a truncated O-glycan epitope, and with CD146 (MCAM/MUC18) may also influence cancer progression and metastasis, by exposing cell surface adhesion molecules [177] and inducing secretion of metastasis-promoting cytokines from vascular endothelial cells [178], respectively. Galectin-4 has been implicated in prostate cancer progression and advanced metastatic disease [179] and may be relevant to predict the metastatic potential of lung adenocarcinoma [180]. Galectin-9-TIM-3 interactions increased the differentiation of myeloid-derived suppressor cells [181]. Additionally, galectin-9 was shown to enhance IFN-γ production by NK cells in a TIM-3-dependent manner [182] and conversely able to impair NK cells function through a TIM-3-independent pathway [183].

The numerous studies on galectin expression have shown associations with poor overall survival, tumour recurrence and metastatic potential. Galectin-binding/inhibiting glycoproteins are being developed for potential application in cancer treatment [184,185] and several clinical trials with different galectin-targeting agents are ongoing, though only on galectin-1 and -3 [186].

7. GLYCOCONJUGATES AS CANCER BIOMARKERS

The altered glycosylation in cancer is of particular interest in the field of biomarker research. Many of the most widely used cancer tests in the clinics detect glycoproteins. The main ones include CA15-3, CA19-9, CA125, CEA and PSA.

Mucins, a family of highly O-glycosylated proteins, can be carriers of these glycan structures. CA15-3 assay is based on the detection of the aberrantly glycosylated MUC1 produced by cancer cells and shed into the circulation. In breast cancer patients, elevated concentrations of CA15-3 have been proved useful for prognosis evaluation in early stage and for monitoring the disease course, including monitoring patients with metastatic disease during active therapy [187–190]. In the absence of readily measurable disease, an increasing CA15-3 concentration may indicate treatment failure [191].

CA19-9 is the most relevant biomarker for pancreatic cancer and the only FDA approved marker for monitoring the disease [192]. CA19-9 corresponds to a SLea antigen that occurs on glycoproteins, such as mucins [193]. It is primarily detected in pancreatic, biliary tract, colorectal and gastric cancers, but it may also be present in patients with other malignancies, such as breast and lung cancers, as well as in non-malignant diseases. CA19-9 serum levels decrease after curative surgery and increase in recurrent disease [194–197].

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CA125 assay for detection of ovarian cancer is based on MUC16 mucin recognition. CA125 concentration in the serum of patients with cancer has been shown particularly useful for evaluation of prognosis and for monitoring purposes. In addition, variation in CA125 levels correlates with regression and progression of the disease. Furthermore, prognosis evaluation benefits from preoperative evaluation of CA125 [188,198]. Recent studies have also reported that CA125 in combination with other markers have good sensitivities for detecting pre-clinical ovarian cancer [199].

CEA is a cell membrane-attached glycoprotein involved in cell adhesion. CEA is normally produced in intestinal tissue during foetal development and its levels drop significantly just before birth. In healthy adults, its serum levels remain very low [200]. CEA is used to monitor the treatment of colorectal cancer patients, although it is also found in patients with other carcinomas (gastric, pancreatic, lung, breast and medullary) as well as in some non-neoplastic conditions [4,200,201].

Despite all the literature supporting the value of these biomarkers for prognostic and monitoring applications, these tests show limited specificity and sensitivity, which preclude their use as screening tools.

A recently introduced glyco-biomarker which has been FDA approved is α1,6-fucosylated α-fetoprotein (AFP-L3). The L3 isoform reactive with Lens culinaris agglutinin is significantly increased in hepatocellular carcinoma patients [202,203]. In addition, the detection of increased levels of serum prostate specific antigen (PSA) is an indication of prostate tumours. However, other benign disorders such as prostatitis, irritation or benign prostatic hyperplasia (BPH) can also lead to increased serum PSA levels. Recent studies have demonstrated increased α2,3-sialic acid in high-risk prostate cancer compared to the low risk patients and BPH individuals [204,205]. Also, differences in the sialic acid content, using Sambucus nigra lectin, were found in metastatic tumours compared to localized tumours [206]. Furthermore, gastric carcinoma expressing SLex has been shown to display more aggressive features and poorer prognosis of the patients [69]. Several studies have also shown correlation between glycan expression and metastasis. For example, it has been demonstrated that the expressing βGal-βGalNAc O-glycan structures is associated with haematogenic and lymphatic spread in breast cancer patients [207].

Emerging fields of research and potential new sources of cancer biomarkers are extracellular vesicles (EVs) [208,209] and circulating tumour cells (CTCs). EVs are released by cells and can be found in body fluids, such as plasma, urine or cerebrospinal fluid. They originate from the plasma membrane or from multivesicular endosomes displaying the particular glycosignature of their parental cells. In this regard, it is important to highlight that most of the typical tumour biomarkers, such as CA15-3, CA19-9, CEA and PSA, have also been detected in EVs [210]. Moreover, a recent study showed that glypican-1 detected in exosomes isolated from serum allowed distinguishing healthy subjects and patients with benign pancreatic disease from patients with early and late stage pancreatic cancer [211,212]. On the other hand, in the context of liquid biopsy, CTCs, which play a key role in metastasis, are perceived as

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a promising tool for tumour biomarker discovery and a non-invasive method for clinical management of tumours [213,214].

All the glycosylated molecules displaying alterations during the malignant transformation have potential applications as cancer biomarkers. Similarly, the glycan binding proteins discussed throughout this review hold also this biomarker potential in the clinical setting. Furthermore, these molecules can be applied as targets for therapy in cancer [215].

The biomarkers field is shifting from tests analysing single targets to multiplexed analysis of numerous proteins with or without post-translational modifications or exclusively glycans. These improvements are possible due to the advances in technologies such as mass spectrometry for glycan analyses and lectin-antibody array methodologies. Overall, a more specific cancer diagnosis will result in earlier disease detection, improved disease monitoring and assistance, tailoring patient-specific therapies.

8. FUTURE PERSPECTIVES

In summary, the understanding of the role of specific glycosylation alterations on specific glycoproteins and their triggered oncogenic mechanisms needs to be considered for either diagnostic or prognostic biomarker purposes, as well as a source for new targets for therapeutic applications. Immunotherapy approaches, such as antibodies or glycan-directed CAR-T cells, targeting tumour-associated glycans or glycopeptides hold major potential for cancer treatment. The recent developments in the field also point towards future applications based on inhibitors either to specific glycosylation-related enzymes or to the blocking of specific glycan recognizing molecules. The knowledge of the glycome of cancer cells and its decisive regulatory effects in cancer biology and progression, together with the interplay of the tumour with the immune response, will set the ground for novel and improved therapeutic strategies for cancer.

Declaration of Interest

The authors declare no conflict of interest.

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Figure 1

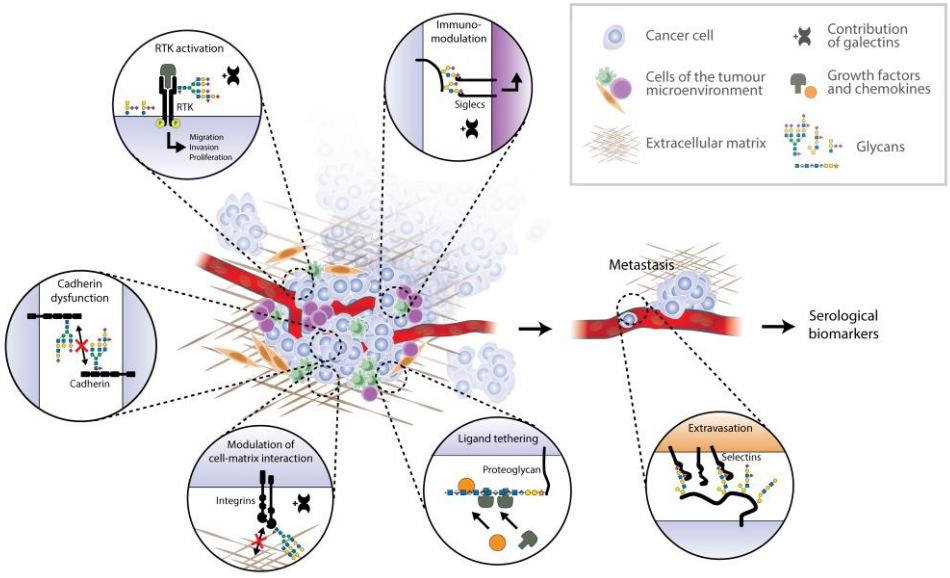

Fig. 1. Selected key functions of glycoconjugates in cancer metastasis. Glycoconjugates contribute by various ways to the hallmarks of cancer and assist in the formation of metastasis.Receptor tyrosine kinases (RTKs) are activated by altered receptor glycosylation, gangliosides and galectin expression, leading to increased cancer cell migration, invasion and proliferation. Cell-cell and cell-matrix interactions are modulated by glycosylation as exemplified by the branched N-glycans of cadherins and integrins, resulting in migratory cancer cell phenotypes. Glycosaminoglycan tether ligands, such as growth factors and chemokines, determine cell signalling processes. Glycan binding proteins, such as siglecs and galectins, regulate the immune response enabling immune tolerance. Sialyl Lewis glycan epitopes are ligands to selectins and contribute to cancer cells and endothelial interactions, facilitating extravasation and in the formation of metastasis. These glycoconjugates can be detected in the serum of patients and thus, be used as diagnostic and predictive biomarkers.

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Figure 2

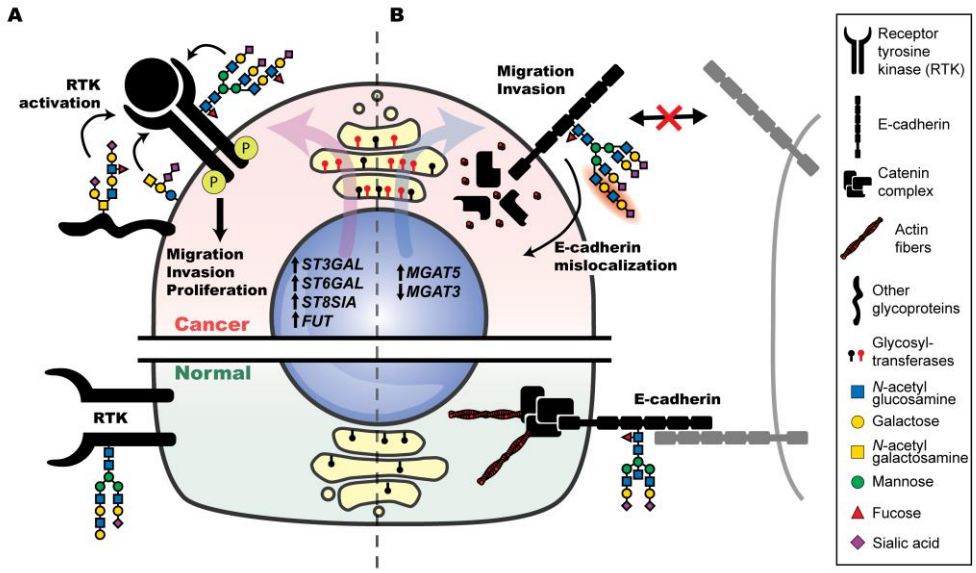

Fig. 2. Changes in glycosylation modify key proteins in cancer cell biology. A. Aberrant glycosylation leads to increased migration, invasion and proliferation through receptor tyrosine kinase (RTK) activation. The increased formation of α2,3-sialylation, α2,6-sialylation and disialogangliosides can be triggered through the overexpression of ST_3GAL, ST6GAL and ST8SIA sialyltransferases, respectively. Additionally, the increased generation of sialofucosylated terminal structures and core-fucosylated N-glycans through altered expression of fucosyltransferases (FUT) is able to promote RTK activation. B. In a normal epithelial cell, E-cadherin functions as an important homophilic cell-cell adhesion molecule. Owing to the increased $MGAT_5$ or decreased $MGAT_3$ expression frequently observed in cancer cells, E-cadherin is predominantly modified with β1,6-GlcNAc branched N-glycans (highlighted in red). The aberrant glycosylation leads to mislocalization and dysfunction of E-cadherin, promoting a more migratory cellular phenotype and contributing to tumour progression.

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