REVIEW

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Glycosylation-related Diagnostic and Therapeutic Drug Target Markers in Hepatocellular Carcinoma

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ABSTRACT

Glycosylation of cell surface proteins regulate critical cellular functions including migration, growth, proliferation, adhesion and apoptosis. Tumorigenic cells possess gene mutations that alter glycosylation enzyme and substrate quantities resulting in glycosylation changes on the surface of the malignant cell. This may lead to metastasis, uncontrolled proliferation and the inhibition of apoptosis all of which are the hallmarks of cancer. The prevalence of hepatocellular carcinoma (HCC) is increasing worldwide, and as a consequence there is a need for improved diagnostic, prognostic and treatment strategies. Currently, the diagnosis of HCC utilises specific glycosylation markers in the serum of patients; however, the efficacy of diagnosis would be further enhanced by including cancer stem cell-specific and novel HCC-associated glycosylation markers. Their application will facilitate earlier, more sensitive diagnoses and reliable staging of the cancer leading to a more effective treatment.

Key words: glycosylation – tumorigenesis – hepatocellular carcinoma – liver progenitor cell.

Abbreviations: AFP: α-fetoprotein; Asn: asparagine; CSC: cancer stem cell; CTC: circulating tumor cells; DOX: doxorubicin; ER: endoplasmic reticulum; 5-FU: 5-fluorouracil; Gal-LMWC: galactosylated low molecular weight chitosan; GPC3: glypican-3; GPAA1: GPI anchor attachment protein 1; HCC: hepatocellular carcinoma; ICAM-1: intercellular adhesion molecule-1; LPCs: liver progenitor cells; GalNAc: N-acetylgalactosamine; GlcNac: N-acetylglucosamine; NLC: nanostructured lipid characters; ppGalNAcT: polypeptide-N-acetyl-galactosaminyltransferase; Pro: proline; Ser: serine; Thr: threonine.

INTRODUCTION

Glycomics is the study of intra- and extracellular carbohydrates associated with plasma membrane proteins, serum proteins or on biomolecules [1]. The importance of glycosylation in cell migration, proliferation and differentiation has led to the utility of these carbohydrate motifs as valuable diagnostic and prognostic markers in liver, breast and lung cancers [2, 3]. Furthermore, glycosylation profiles in mammalian systems also influence the immune response, cell signaling and cell-cell recognition of various cell types [4]. The liver is the main producer of proteins in the serum, and therefore glycosylation changes, due to pathologies within the liver, can easily be characterised and potentially used diagnostically. Already this approach has been used in hepatocellular carcinoma (HCC) diagnosis by detecting glycosylation profiles on the serum protein α-fetoprotein (AFP). In adults, AFP is typically only present in the serum of HCC patients [3].

The prevalence of liver cancers, of which 90% are HCCs, is increasing annually by 3.7% in men and by 2.9% in women [5]. Furthermore, the 5-year survival rate for all liver cancers is only 16.6% [5]; in un-resectable liver HCC, this figure drops to 5% [6]. This poor prognosis is in part due to late diagnosis of liver cancer; however, even with an early diagnosis only 20% are responsive to standard chemotherapies [7]. A trend analysis for liver cancer indicates a significant rise in death rates of this pathology [5]. With liver cancer-associated factors such as obesity, hepatitis B and C, alcoholism and diabetes all increasingly prevalent, there is a need for more efficient diagnosis and targeted treatments.

LIVER PROGENITOR CELLS

In acute liver injury, liver homeostasis is maintained by hepatocyte regeneration through self-replication. Although 80% of the liver is comprised of hepatocytes, the dominant cell type [8] in chronic liver injury, hepatocyte self-replication is not sufficient to enable necessary liver compensatory growth. Consequently, liver progenitor cells (LPCs) are required to facilitate complete liver regeneration [9].

Normally, LPCs are quiescent and present in low numbers surrounding the periportal region in the canals of Herring [9]. In a healthy individual these cells account for less than 1% of all cells in the liver [10]. This number dramatically increases in the setting of chronic liver injury. Liver progenitor cells are bi-potential and thus capable of differentiating into hepatocytes and cholangiocytes to regenerate the liver [9]. To demonstrate the presence of LPCs *in vitro*, the cell surface marker PanCK can be utilised [10].

Therapeutic potential of liver progenitor cells

Hepatocellular carcinoma is the 6th most diagnosed cancer [5] and the 3rd highest cause of cancer-related death worldwide [3]. Unfortunately, HCC is frequently resistant to standard chemotherapies [11], and with limited organ donation rates, liver transplantation is an inadequate treatment option. Consequently, there is a need for novel liver cancer therapies. A promising alternative is stem cell transplantation in which stem cells may induce liver regeneration of end-stage liver disease allowing the patient time until a suitable transplant donor is identified [12].

The utility of mature hepatocytes for cell transplantation is hindered by their inability to proliferate and retain function when subjected to long term *in vitro* culture [9]. Consequently, LPCs may have potential as therapy for liver pathologies due to their stem-cell like ability to expand and differentiate to regenerate the liver [13]. In contrast to hepatocytes, LPCs are able to rapidly differentiate into functional cells and are easily expanded *in vitro* for maintenance in long-term cultures [9].

Liver progenitor cells have assumed increased significance since their involvement in liver pathologies was described with evidence suggesting that LPCs can predispose patients to developing HCC [14]. Initial studies proposed that LPCs are the cellular targets for tumorigenic transformation in the development of HCC [14]. As such, LPC lines have the potential to be tumorigenic if they are maintained in extended culture [15]. Altered glycosylation markers on the surface of these malignant LPCs could identify and facilitate isolation of these tumorigenic cells. Ultimately, particular glycosylation profiles may improve diagnoses and be used to track cancerous cells *in vivo* [16].

GLYCOSYLATION

Glycosylation is a co- or post-translational protein modification which can affect cell properties including adhesion, trafficking, signaling, mediate aspects of the innate immune system and influence host-pathogen interactions and protein folding [16-17]. Glycosylation is the process of forming oligosaccharide chains known as glycans, and occurs on many proteins, both soluble and membrane-bound (Fig. 1). This process is enzyme–directed and site specific [18]. Two main protein glycosylation pathways exist, namely N-linked and O-linked. Altered protein glycosylation has been linked with disease pathologies such as cancer, e.g. HCC. Further study of these disease-risk glycosylation patterns has the potential to improve cancer diagnosis, staging, prognosis and treatment [18]. The glycans found on proteins have also been used as oncogenic markers for tracking cells *in vivo* or isolation *in vitro* [16].

N-glycosylation

N-glycosylation is the most common glycosidic bond in nature [19] and is defined by the attachment of a carbohydrate to an asparagine (Asn) moiety in a protein [17]. Biosynthesis of N-glycans consists of three major steps that occur consecutively in the endoplasmic reticulum (ER) and the Golgi apparatus.

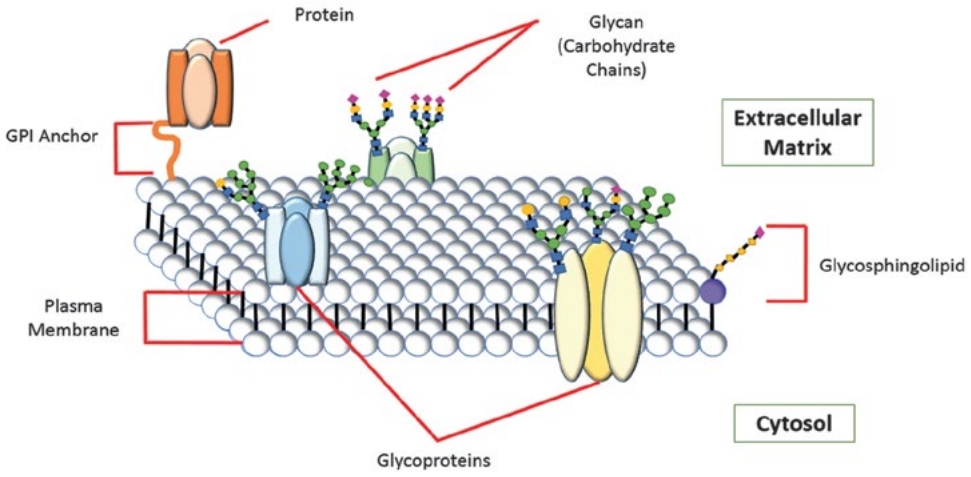

Fig. 1. Glycoproteins on the cell surface of a eukaryotic plasma membrane. Proteins protruding from the eukaryotic plasma membrane are covered with carbohydrate motifs of varying length and complexity. Cell surface glycans function as modulators of cell signaling and cell-cell adhesion.

Oligosaccharides that require the Asn-linkage are initially assembled as a common precursor of Glc3Man9GlcNAc2 [20]. This complex is attached to a dolichol lipid, which acts as a carrier molecule [11]. *En bloc* transfer of this glycan to the nascent protein by an oligosaccharyltransferase multisubunit complex occurs within the endoplasmic reticulum (ER) [17].

N-glycan-polypeptide hybridisation is highly site-specific, occurring via the GlcNAc-β-Asn link where the Asn residue is positioned adjacent to an X-serine/threonine (Ser/Thr) [21]. Following covalent attachment, the N-glycan is then modified by glycosidases that cleave three glucose residues, and a minimum of one mannose residue from the glycan chain in the ER, thus allowing quality control of the correctly folded protein and subsequent release from the ER. Additional mannose residues may be removed in the first compartment of the Golgi apparatus [20]. Subsequent decorations of the glycan structure within the Golgi apparatus is undertaken by the action of glycosyltransferases that attach discrete monosaccharide units. Although N-glycan modification can be diverse, a Man3GlcNAc2-Asn core is conserved (Fig. 2A) thus N-Glycans can be divided into three structural groups: high mannose, complex and hybrid [17] (Fig. 2B).

O-glycosylation

O-glycosylation is predominantly characterised by the attachment of carbohydrates to Ser or Thr residues on proteins [17]. Mucins are the most abundant class of O-glycans (a class of O-glycosylation, termed O-GlcNAc is also found within eukaryotes but will not be discussed further) defined by their strong water binding capacity [22]. This is due to numerous sialyated O-glycans attached to clusters of Ser or Thr residues.

O-glycosylation in general is initiated by the transfer of N-acetylgalactosamine (GalNAc) from UDP-GalNAc to either the Ser or Thr residue. This transfer is catalysed by one of more than 21 polypeptide-N-acetyl-galactosaminyltransferase (ppGalNAcT) enzymes and occurs in the Golgi apparatus [23]. The expression, timing and type of ppGalNAcT effects the variety and number of O-glycans present in each tissue [20]. Interestingly, a proline (Pro) residue adjacent to the sites of GalNAc addition occurs more frequently in mucin O-glycosylation than other amino acids [24]; however, unlike N-glycosylation no consensus sequence is required. In addition, no glycosidases are involved in the final processing of O-glycans within the Golgi apparatus [17].

There are eight O-glycan core structures (Core 1-8) (Fig. 3) that have been identified within mammalian systems, with Core 1 and 2 being the most common. In general, the addition of sugars, such as fucose, to the core structure results in a variety of O-glycan structures. The Core 1 base structure is synthesised by the action of β1-3 galactosyltransferase to GalNAc [25], while Core 2 structures are formed by the addition of N-acetylglucosamine (GlcNac) to GalNAc in the Core 1 glycan. In contrast to the N-glycosylation pathway, O-glycan sugars are added to the core stepwise as the GalNac residue is attached to the protein within the Golgi.

Functions of glycoproteins

Glycans are central to mammalian development by acting as ligands for cell receptors, facilitating cell recognition and cell-to-cell adhesion [2, 17]. This activity facilitates antigenic determination and modifies the immunogenicity of cells and tissues [26]. Furthermore, the stability of hydrogen bond interactions between proteins and glycans alters the protein structure conformation. In particular, the activity of certain proteins such as mucins, that effect signal transduction between cells, is modulated by O-glycans [17]. Importantly, these mucins are capable of forming a protective, hydrating layer to the underlying epithelial tissue [22].

GLYCOSYLATION CHANGES IN CANCER

Altered glycosylation profiles have been associated with many cancers including liver, breast and lung [2, 3]. Regulators of cellular proliferation, survival and differentiation are all influenced by glycosylation [27]. Certain gene mutations can facilitate aberrant glycosylation in tumorigenic cells that consequently have a downstream effect on cell-surface glycan

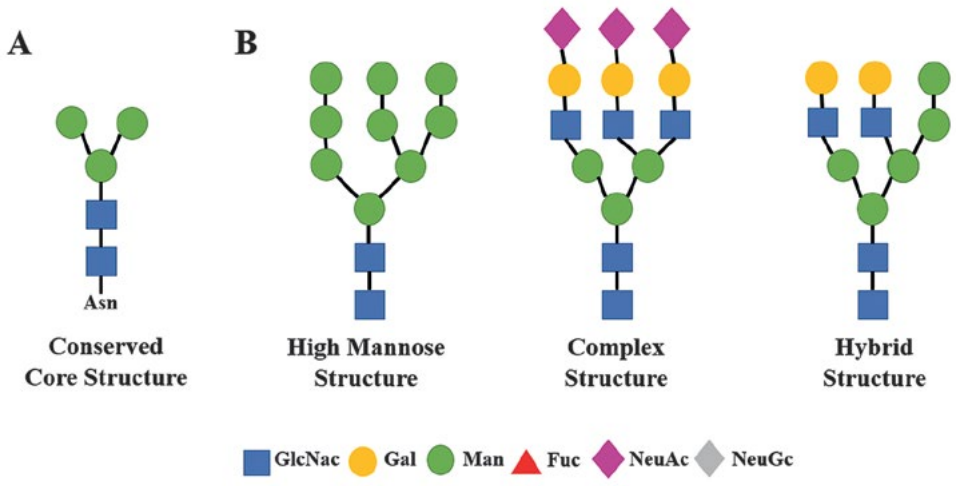

Fig. 2. Structural classes of mammalian N-glycans. (A) The conserved N-glycan core structure, (B) the three structural groups of glycans, each group being defined by the composition of the carbohydrate; high mannose, complex or hybrid.

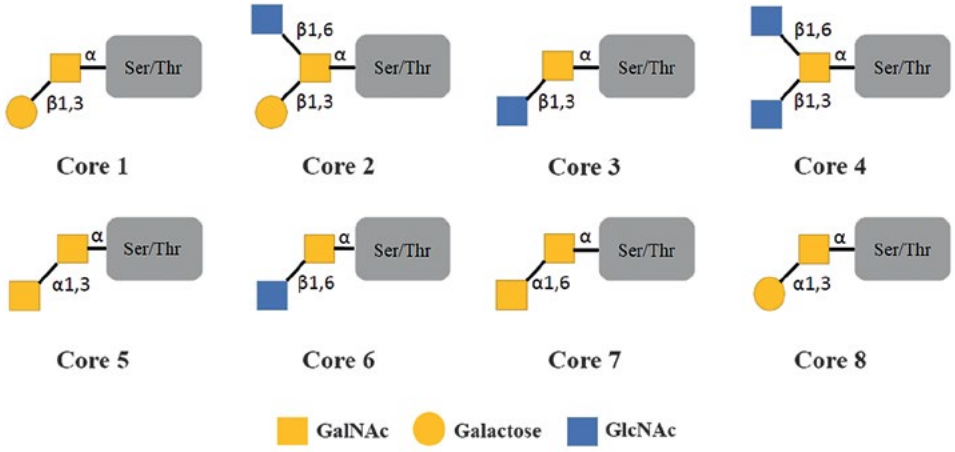

Fig. 3. O-Glycan structures Core 1-8. Core 1 and 2 are the most abundant out of the eight identified mammalian Core structures. Core 1 is composed of a galactose and is attached to the base N-Acetylgalactosamine (GalNAc). Core 2 utilises the Core 1 complex with an addition of N-Acetylglucosamine (GlcNAc) to the GalNAc. Cores 3-8 are synthesised in a similar method.

structures. However, the most frequent glycome alteration found in human diseases and cancers [28] is due to the loss of protein-complexes and chaperones. These glycosylation alterations can be divided into different groups based on the type of oligosaccharide added to the glycan chain. The three most common changes associated with HCC are discussed below.

Galactosylation

The metastatic potential of cancer cells within the primary liver lesion can be regulated by glycosylation of cell surface proteins via genes *GNTV* and *GNTIII* [29]. Within the Golgi apparatus GNTV competes with GNTIII for oligosaccharide substrates [30]. Here an increase in GNTV activity affects the formation of multi-antennary N-glycans [31]. Consequently, this change in the N-glycan affects the adhesive properties, cell motility, migration potential and loss of contact inhibition of the cell [30]. For example, an HCC cell line, HepG2, demonstrated that high levels of *GNTV* gene expression were directly associated with increased metastatic potential [18]. Cell endocytosis is also mediated by *GNTV* as this protein blocks EGF and TGF-β receptor endocytosis [32]. In addition, the *GNTV* promoter has *Ets* transcription factor binding elements that in response to signals from mediators of proliferation, induces its transcription [33]. Therefore, malignant cells with uncontrolled proliferation can modulate *GNTV* expression.

Hepatitis B virus-encoded (HBx) protein is considered to be a HCC risk factor [34]. In HCC patients GalTI was demonstrated to be up-regulated by HBx [35]. Therefore, HBx may increase growth in hepatomas thus influencing development and progression of HCC [35]. It has been suggested that GalTI is up-regulated in metastatic tumors as well as being directly associated with migration, adhesion and invasion of tumor cells in other cancers [16, 35].

Fucosylation

Core fucosylation of glycans can affect protein structure and folding due to steric effects of glycan-lectin binding via chaperones before being moved from the ER to the Golgi apparatus [36]. Consequently, cell migration, growth and tumor invasion controlled by glycan-lectin binding are affected [37]. The *FUT8* gene, which controls core fucosylation, can influence the proliferation [38] and migration of tumour cells as it affects cell adhesion molecules. For example, Ji et al. (2013) reported that *FUT8* knockdown reduced proliferation, invasion and migration of human HCC cell lines [39]. Interestingly, Zhao et al. (2008) found that sera derived from HCC patients had elevated core fucosylation and that this elevation was also present in liver tumorigenic tissue [2].

In HCC an up-regulation of *FUT8* transcription correlates with increased production of α-1,6 fucosyltransferase which adds fucose to N-linked glycoproteins [18]. A similar increase in GDP-fucose levels has been reported in HCC tissue [38]. Although an up-regulation of *FUT8* is necessary to generate fucosylated AFP, this process does not account for all fucosylated AFP seen in HCC [39]. Consequently, it is considered that GDP-fucose is an important regulatory factor in HCC-associated AFP fucosylation [40]. Furthermore, the *FUT* gene family, consisting of eleven genes (*FUT1* to *11*), also affects the expression of Lewis (Le) antigen found on glycolipids in epithelial cancers [41]. A contradictory study demonstrated that FUT genes 1-7 and 9-11 produce α-1,3 fucosylated tri-antennary glycans in the HCC liver of patients with a background of liver cirrhosis or fibrosis [42]. However, core α-1,6 fucosylated bi-antennary glycans, under regulation by *FUT8*, were elevated in cirrhosis patients only [42]. In a subsequent study, Ji et al. (2013) did observe higher levels of *FUT8* expression in cancerous tissue than in adjacent non-cancerous liver tissue [38].

Sialyation

Sialyation prevents oligosaccharide antenna from being extended and is typically found at the non-reducing terminal position of the glycan [43]. Sialyltransferase genes *ST3GalI* and *ST3GalV* regulate cell adhesion of HCC cells. Consequently this alteration to a glycan structure is mediated by these proteins which is associated with cancer metastasis [44].

ST6GalI is commonly up-regulated in cancer [45] with the functional roles of this protein including modulating N-glycans

cell surface receptors [46], promotion of cell migration and tumor invasiveness [47]. Importantly, ST6GalI is a potent negative regulator of galectin-dependent apoptosis [48]. Subsequent experiments by Dall'Olio et al. (2004) revealed that the expression of the gene was altered in some cases of HCC, but not in cirrhotic patients [49]. In normal epithelial tissue, *ST6GalI* expression is present only in stem and progenitor cells [45]. However, in tumorigenic tissue high levels of *ST6GalI* gene expression have been linked to the expression of known cancer stem cell (CSC) markers CD133 and ALDH1 [45]. These CSCs have been previously associated with a high recurrence rate in cancer [50]. For example, tumorigenic LPCs, which are potential sources of HCC, have been correlated with poor prognosis and recurrence [51].

O-glycosylation of membrane proteins in HCC

Further examples of O-glycosylation of proteins exist including those attached to glycosylphosphatidylinositol (GPI anchored). Alteration of these glycolipids has been connected to HCC. A recent study by Zhu et al. (2014) identified an increase in the expression of glycolipids compared to the non-tumour control [52]. GPI anchors have also been associated with tumorigenic cell behaviour [53]. GPI anchor attachment protein 1 (GPAA1) RNA expression was up-regulated in HCC cells located at loci 8q24.3, which is frequently amplified in HCC cells [54]. Up-regulation of GPAA1 is associated with increased proliferation rate and cell adhesion ability as well as poor cellular differentiation, aggressive tumour development and metastasis [53].

GLYCOSYLATION DIAGNOSTIC MARKERS IN HCC

Currently, the diagnosis of HCC utilises glycosylation markers such as fucosylation on the surface of AFP in blood serum. An increase in the expression of the fucosylation regulator, *FUT8*, has been associated with increased levels of core fucosylation in hepatic tissue and the sera of HCC patients [55]. The α-1,6 fucosylation of AFP is said to distinguish HCC from other liver pathologies such as chronic liver disease [56]. However, the presence of fucosylated AFP can fluctuate due to normal physiological events [57]. Interestingly, up to 40% of HCC patients have no serum AFP [58] at diagnosis as well as throughout the patient's life [59]. Therefore, the AFP detection assay is not conducive to an early diagnosis due to a high false negative rate [60].

POTENTIAL GLYCOSYLATION MARKERS IN HCC

The identification of novel HCC markers may improve the diagnostic accuracy of clinical evaluations of individuals with suspected HCC thus leading to novel therapeutic targets which will improve our understanding of the biological classification of HCC. This current review highlights seven potential markers which may be used in future studies relating to HCC.

CD147

This protein is transmembrane in nature and its abundance is increased on the surface of HCC cells [61], which is positively correlated with HCC progression and prognosis. In HCC, CD147 is associated with the stimulation of adjacent tumour cells and fibroblasts, which, in turn, induce an increase of several extracellular matrix metalloproteinases [62]. This glycoprotein has also been associated with multi-drug resistance and angiogenesis of HCC [63]. Several monoclonal antibodies have recently been produced to target and silence CD147, with some promising results, such as decreasing cell growth and metastasis of HCC cells *in vitro* [64].

ICAM-1

Intercellular adhesion molecule-1 (ICAM-1), a single-chain glycoprotein, has been suggested as a potential prognostic marker for AFP-negative HCC patients [65]. Currently, studies have identified this biomarker as an indicator of malignant cell metastasis, development and invasion [66]. Furthermore, increased ICAM-1 expression in the sera of HCC patients was shown to be positively correlated to high levels of metastasis [67]. Recently, Liu et al. (2013) found that ICAM-1 mediates metastasis by increasing the survival of natural killer cell-resistant circulating tumor cells (CTCs) [68].

GNTV

GNTV expression determines GlcNAc branching of cell-surface proteins [30] and has been directly associated with the metastatic potential of the tumorigenic cell and may be useful as a prognostic marker in HCC for tumor invasiveness [69]. Recently, GNTV has been associated not only with the initial stages of cancer, but also serves as a prognostic tool for progression to advanced stages of cancer and metastasis [70]. Increases in the number of N-glycan antenna mediated by GNTV expression were correlated with hepatocarcinogenesis [71]. However, GNTV may represent a novel diagnostic marker for HCC given that GNTV activity within the serum of HCC patients was higher than chronic liver disease patients compared to normal controls [79]. Furthermore, GNTV has been positively correlated with the percentage of fucosylated AFP [79].

GP73

A study by Block et al. (2005) identified a fucosylated Golgi protein 73 (GP73) that was more abundant in the serum of human HCC patients than in normal controls [72]. Currently, detecting GP73 in the serum of patients is considered more sensitive than the standard diagnostic assay for AFP; in particular in those with low AFP [73]. GP73 has been shown to be 2-3 times more sensitive than using AFP alone [74]. However, the predictive value of GP73 in HCC patients has recently been questioned, as the assay cannot distinguish between malignant and benign cancer of the liver [75].

Haptoglobin glycoforms with alterations in fucosylation and sialyation are specific to HCC and tumor progression [76]. An increase in fucosylation on haptoglobin is associated with HCC patients that have a background of hepatitis B virus (HBV) and alcohol related liver cirrhosis when compared to cirrhosis with other etiologies [77]. Furthermore, an increase in the bi-fucosylation of haptoglobin was related to HCC of all etiologies compared to cirrhosis and can indicate early stages of HCC in a cirrhosis background. This approach was

shown to be more reliable when combined with AFP rather than standing alone [78]. A subsequent study conducted by Zhang et al. (2011) confirmed this increase in fucosylation of haptoglobin [79]. This was associated with an increase of tri-antennary glycans and a decrease in bi-antennary structures on the haptoglobin β chain in HCC patients compared to that of liver cirrhosis patients. This data shows that novel glycoprotein markers can be used collectively as oncogenic markers for HCC diagnosis and progression.

Fucosylated-hemopexin

Core fucosylation of bi-antennary glycans have been detected on hemopexin, a plasma glycoprotein, in the serum of HCC patients [80]. Evaluated on its own, fucosylated hemopexin had both a sensitivity and specificity of 92% when HCC patients' sera were analysed [81]. Fucosylated hemopexin is therefore a potential candidate for HCC diagnosis as it is more abundant in the sera of HCC patients as compared to normal controls [82]. A study by Kobayashi et al. (2012) confirmed that fucosylated hemopexin was a candidate biomarker for HCC [83]. However, this marker can only indicate a carcinogenic liver given that no relationship was found between the glycoprotein and tumor progression [83].

GPC3

Glypican-3 (GPC3) is part of a subclass of membrane-bound proteoglycans involved in differentiation, migration and cellular growth [84]. This marker has been used to distinguish between non-malignant liver disease and other forms of liver cancer [85]. Combined with other biomarkers including AFP, GPC3 is able to improve the accuracy of HCC diagnosis [86]. Glypican-3 can also be used to detect early-stage HCC with only small tumours, given that the marker is already expressed [87]. When utilising only the AFP marker, the detection sensitivity decreases by 20-40% [88].

GLYCOSYLATION MARKERS AS THERAPEUTIC DRUG TARGETS

In addition to diagnostic and prognostic applications, glycosylation markers may represent suitable targets for monoclonal antibodies to deliver therapeutic drugs. Cancer stem cells, like LPCs, are known to be resistant to chemotherapy and radiotherapy, and in many cases lead to high recurrence rates [89]. Consequently, the CSC marker and CD123, amongst others, would be ideal as therapeutic targets. Alternatively, glycosylation pathways that promote metastasis, proliferation and inhibit apoptosis may also be suitable targets for novel drugs. For example, Jin et al. (2009) targeted the highly glycosylated α-chain of CD123 with a monoclonal antibody, which successfully increased survival time of mice with acute myeloid leukemia [90].

Glycosylation of nanoparticles has also been utilised to target and deliver therapeutic drugs to malignant cells. Galactosylated low molecular weight chitosan (Gal-LMWC) nanoparticles carrying doxorubicin (DOX), an anti-cancer drug, specifically targets hepatocytes [91]. Gal-LMWCs act by entrapping the cationic DOX in order to target HepG2 cell lines *in vitro* [92]. Docetaxel-loaded hepatoma-targeted solid lipid nanoparticles were also shown to have no adverse effects on healthy and fibrotic livers, based on histology [93]. This was confirmed by treatment with an alternate chemotherapeutic drug, 5-fluorouracil (5-FU), which showed increased cytotoxicity in HepG2 cell lines due to the galactosylated nanostructured lipid characters (NLC) [94]. Potentially, galactosylated NLC could reduce the 5-FU concentration needed to combat HCC given that cytotoxic levels were halved using Gal-NLC. Microspheres coated in galactosyl-chitosan showed a slower release compared to uncoated microspheres, suggesting that a galactosyl-chitosan coating is ideal for targeted delivery [112].

CONCLUSION

The increasing prevalence of risk factors such as hepatitis infections, diabetes and obesity means that the incidence of HCC will continue to rise in the future. The current application of glycosylation markers in HCC diagnosis and treatment is promising, but limited. There is a need for more extensive characterisation of glycosylation changes associated with carcinogenesis to identify novel biomarkers. This needs to be accompanied by a better understanding of the basis of these changes and their relation to the cancer phenotype. The application of this knowledge in both diagnosis and treatment will lead to improved outcomes for patients with HCC.

Conflicts of interest: The authors report that there were no conflicts of interest.

Authors' contribution: M.W.: drafting manuscript, critical revision; L.D.: critical revision and response to reviewer's recommendations; K.S.: critical revision; G.Y.: experimental design, critical revision.

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