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Glypicans as Cancer Therapeutic Targets

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Abstract

Glypicans are a group of cell surface glycoproteins in which heparan sulfate glycosaminoglycan chains are covalently linked to a protein core. The glypican gene family is broadly conserved across animal species and plays important roles in biological processes. Glypicans can function as co-receptors for multiple signaling molecules known for regulating cell growth, motility and differentiation. Some members of the glypican family, including glypican 2 (GPC2) and glypican 3 (GPC3), are expressed in childhood cancers and liver cancers, respectively. Antibody-based therapies targeting glypicans are being investigated in preclinical and clinical studies, with the goal of treating solid tumors that do not respond to standard therapies. These studies may establish glypicans as a new class of therapeutic targets for treating cancer.

Keywords

glypican; Wnt signaling; liver cancer; childhood cancer; antibody-based therapy

Glypicans as Potential Cancer Targets

Glypicans are proteoglycans with heparan sulfate (HS) chains that are linked to the cell surface via a glycosylphosphatidylinositol (GPI) anchor. Glypicans have the ability to

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Conflicts of Interest:

The National Cancer Institute (NCI) holds patent rights to anti-GPC2 and anti-GPC3 antibodies in many jurisdictions, including the United States [e.g., U.S. Patent 9,409,994, U.S. Patent 9,206,257, U.S. Patent 9,304,364, U.S. Patent 9,932,406, U.S. Patent Application 62/716,169, and U.S. Patent Application 62/369,861], China, Japan, South Korea, Singapore and Europe. Claims cover the antibodies themselves, as well as conjugates that utilize the antibodies, such as recombinant immunotoxins (RITs), antibody drug conjugates (ADCs), bispecific antibodies and modified T cell receptors (TCRs)/chimeric antigen receptors (CARs), and vectors expressing these constructs. Anyone interested in licensing these antibodies can contact Dr. Mitchell Ho for additional information.

Resources

regulate cellular morphology and cellular behaviors including survival and differentiation; thus, it is not surprising that changes in glypicans expression have been reported in various human cancers. The abnormal expression of glypicans regulates tumor proliferation and progression by modulating Wnt [1, 2], hedgehog (Hh) [3] and other signaling pathways.

Selecting appropriate targets in solid tumors is challenging. Recent studies show that glypican 2 (GPC2) [4, 5] and glypican 3 (GPC3) [6] are highly specific for tumor tissues. More importantly, GPC2 and GPC3-targeted antibody-based therapies have eliminated established tumors in preclinical studies, and unleashed the potential of targeting glypicans for cancer therapy.

Expression of Glypicans in Cancer

Glypicans are predominantly expressed during embryonic development. Since they regulate a variety of growth and survival factors during morphogenesis, recent reports indicate that changes in glypican expression may be associated with tumorigenesis and progression of cancers.

GPC1

Expression of GPC1 has been linked with proliferation, angiogenesis and metastasis of pancreatic cancer cells [7, 8]. GPC1 is overexpressed in breast cancer and modulates the effects of heparin-binding growth factors in breast cancer cells [9]. The expression of GPC1 is also upregulated in esophageal squamous cell carcinoma (ESCC) and correlated with poor survival [10]. Additionally, GPC1 overexpression is found in gliomas and might play a role in angiogenesis [11, 12].

GPC2

GPC2 is one of several mRNA transcripts highly expressed in multiple childhood cancers, including neuroblastoma [13]. An ideal therapeutic target may have limited expression on normal human tissues. Recent studies demonstrate that the expression of GPC2 protein is significantly increased in neuroblastoma and undetectable in normal tissues, including the brain, heart, lung and kidney, indicating that GPC2 is a suitable tumor antigen in neuroblastoma [4, 5].

GPC3

GPC3 is expressed in over 70% of hepatocellular carcinoma (HCC) [14], the most common type of liver cancer accounting for approximately 90% of all cases [15]. Notably, its expression is not detected in non-malignant tissues [16]. Moreover, GPC3 expression is correlated with poor prognosis in HCC patients [17]. Thus, GPC3 is a suitable biomarker and prognostic factor of HCC, and an attractive target for HCC therapy. The overexpression of GPC3 has also been reported in hepatoblastoma, lung squamous cell carcinoma (LSCC), testicular and ovarian yolk sac tumors, melanoma, ovarian clear cell carcinoma and other cancers [18–23].

The most common isoform of GPC3 is isoform 2, which encodes a 70-kDa core protein with 580 amino acids; additionally, three other variants have been detected that encode alternative spliced forms [24]. Given these findings, expression analysis of GPC3 (or other glypicans) isoforms in normal tissues and cancers may be important for the development of therapeutic intervention or diagnostics in the era of precision medicine.

In addition, several studies revealed significant intratumoral heterogeneity of GPC3 expression levels in HCC tissues [25–27]. These observations raise a critical issue that the immunoreactivity found in a small needle biopsy specimen may not represent the overall level of GPC3 expression within tumor. Thus, acquisition of multiple samples from different regions of tumor sites are important to understand the heterogeneous expression of glypicans in cancer. The impact of such diversity on clinical outcomes such as response to glypican-targeted therapies remains largely unknown and is an area of research worthy of intensive efforts.

Expression of GPC3 may also inhibit cell proliferation. . A loss-of-function mutation in *GPC3* causes Simpson-Golabi-Behmel syndrome (SGBS), an X-linked disorder characterized by pre- and post-natal overgrowth. GPC3-deficient mice (*GPC3^{-/-}*) also display similar phenotypic features of SGBS [28]. In GPC3 transgenic mice, overexpression of GPC3 in hepatocytes suppresses hepatocyte proliferation and liver regeneration [29].

GPC4/5/6

Similar to GPC1, an increased expression of GPC4 has been reported in pancreatic cancer [30]. GPC5 is overexpressed in rhabdomyosarcoma and stimulates rhabdomyosarcoma cell proliferation [31–33]. However, GPC5 expression is downregulated in breast and prostate cancer [34, 35], indicating GPC5 may have distinct roles in different cancer types. Although one report suggests that GPC5 is upregulated in non-small cell lung cancer (NSCLC) [36], more studies demonstrate that GPC5 is a tumor suppressor in NSCLC and low expression of GPC5 is associated with poor survival in lung adenocarcinoma [37, 38]. Additionally, GPC6 expression is significantly higher in gastric cancer tissues compared to that in normal tissues [39]. Overexpression of GPC6 has also been detected in ovarian cancer [40].

To summarize, glypicans are expressed in abnormally high levels in various types of cancers, suggesting that cell surface glypicans may emerge as a new group of therapeutic targets in cancer.

Glypicans in Cancer

Glypicans are essential components of the extracellular matrix (ECM). They are involved in many important biological and pathological processes by mediating cell-ECM and cell-cell interactions. The most significant characteristic of glypicans is that they contain various numbers of HS chains close to the cell membrane; this unique structure affords glypicans the ability of to rescue and sequester different growth factors, morphogens, chemokines and cytokines. Glypicans concentrate these factors and form a gradient around the ECM and cell membrane, which facilitates the recognition of cell membrane receptors with reduced threshold. In addition, glypicans specifically bind to several factors through the identified

domain within their HS chains as well as the core protein. In this sense, glypicans act as essential co-receptors and trigger the activation of intracellular signaling including Wnt, Hh, FGF, and many other signaling pathways.

Wnt Signaling

Wnt signaling plays pivotal roles in many biological and pathological processes, including embryonic development, differentiation, cell polarity and tumorigenesis. It has been demonstrated that glypicans act as Wnt modulators in cancer. For example, GPC2 can positively regulate canonical Wnt signaling in neuroblastoma, as silencing GPC2 inactivates Wnt/ β -catenin signaling and reduces the expression of target genes [5]. Although GPC3 is a tumor-promoting gene in HCC by activating Wnt/ β -catenin signaling [24, 41, 42], GPC3-induced regulation on Wnt signaling is based on the specific cellular environment. Studies showed that depletion of GPC3 inhibited non-canonical Wnt/JNK signaling, while concomitantly activated canonical Wnt/ β -catenin signaling [28]. In murine mammary adenocarcinoma cells, GPC3 inhibits canonical Wnt signals that are involved in cell proliferation and survival, whereas activates non-canonical Wnt signals that determine cell morphology and migration [43]. Moreover, a recent study has shown that GPC4 is co-localized with different Wnts to lipid raft and non-lipid raft microdomains and thereby regulates distinct Wnt signaling pathways [44]. In lung adenocarcinoma and prostate cancer, GPC5 inhibits Wnt/ β -catenin signaling to suppress tumor cell proliferation, migration, invasion and epithelial-mesenchymal transition (EMT) [38, 45, 46]. GPC6 promotes tumor cell growth and invasiveness by inhibiting canonical Wnt signaling and activating Wnt5a/JNK/p38 MAPK signaling [47].

GPC3 interacts with both Wnt and frizzled (FZD) to form a tripartite complex and triggers downstream signaling (Figure 1). Both the core protein and the HS chains may be involved in GPC3-modulated Wnt signaling. Previous reports indicate that GPC3 HS chains may not be required for Wnt binding [41], but are essential for interaction with FZD [2]. Our studies identified a Wnt-recognizing domain on the HS chains of GPC3 using a HS-specific antibody that blocks GPC3 and Wnt3a interaction [1, 48]. This unique Wnt-binding region is at least four disaccharides in length with 6-O sulfation or shorter when 3-O sulfation is present. Sulfatase 2 (SULF2), an enzyme with 6-O-desulfatase activity is upregulated in HCC. Desulfation by SULF2 releases HS-stored Wnts and may activate canonical Wnt signaling. In addition, we and others have shown that the core protein of GPC3 without HS also bound to Wnt [41, 49], although further structural and functional studies will be necessary to reveal the precise Wnt binding domain on the protein core of GPC3 or other glypicans.

R-spondin (RSPO) proteins emerged as important growth factors that drive the renewal of epithelial stem cells in many adult vertebrate tissues [50]. They amplify target cell sensitivity to Wnt ligands by increasing Wnt receptor levels [51, 52]. Translocations and mutations in regulators of RSPO have been reported in multiple cancers (e.g. colon cancer) [53, 54]. More importantly, a recent study has shown that GPC4 and GPC6 are required for RSPO-enhanced Wnt signaling in response to physiologically relevant low levels of Wnt [55].

RSPOs can potentiate Wnt signals through interactions between HS chains and the thrombospondin type 1 (TSP)/basic region (BR) domains of RSPOs [56].

Hedgehog (Hh) Signaling

Glypicans exhibit opposing roles in Hh signaling, which is important for embryonic morphogenesis [3, 57]. Among these roles, GPC3 is a potent inhibitor of Hh signaling (Figure 2). Loss of function mutations of *GPC3* causes SGBS, partially due to hyperactivation of the Hh pathway [58, 59]. GPC3 binds to sonic hedgehog (Shh) and indian hedgehog (Ihh) with high affinity in a HS-independent manner, resulting in a strong competition with Patched (Ptc) for Hh binding [60]. The HS chains of GPC3 are required for low-density-lipoprotein receptor related protein-1 (LRP1) mediated-glypican/Hh complex endocytosis [61]. GPC5 and GPC6 show stimulating effects on Hh activation. In contrast to GPC3, GPC5 binds to both Hh and Ptc through its glycosaminoglycan chains and promotes Hh signaling in rhabdomyosarcoma cells, possibly by facilitating or stabilizing the interaction between Hh and Ptc1 [31]. Recently, GPC6 has been reported to stimulate Hh signaling by interacting with Hh and Ptc1 through its core protein and glycosaminoglycan chains, respectively [62].

Fibroblast Growth Factor (FGF) Signaling

FGF is one of the heparin-binding growth factors. Many studies reveal that glypicans modulate FGF signaling during tumor cell proliferation. In breast and pancreatic cancer, GPC1 is upregulated and promotes tumor mitogenic signaling by modulating many heparin-binding growth factors, including FGF2 [7, 9]. There is evidence demonstrating GPC1 cooperates with type V collagen to concentrate FGF2 at the cell-ECM interface, thereby affects ECM stability and tumor cell proliferation [63]. GPC1 also contributes to the enhanced signaling of FGF-2/FGFR1c in glioma cells, likely due to highly elevated 2-O-sulfation and 6-O-sulfation-containing disaccharides [12]. Additionally, GPC1 is upregulated in glioma vessels, causing enhanced FGF signaling and glioma angiogenesis [11]. In GPC1 knockout KRAS-driven genetic mouse model of pancreatic ductal adenocarcinoma, GPC1^{-/-} tumor cells show less invasive properties in response to FGF2 treatment compared to tumor cells derived from wild type mice [8]. Silencing of GPC4 results in attenuated DNA synthesis in response to FGF2 treatment in HEK293T cells [64]. Lastly, GPC5 increases rhabdomyosarcoma cell proliferation by enhancing the intracellular FGF2 signaling and altering the cellular distribution of FGF2 [33].

Cancer Therapies Targeting Glypicans

Therapies Targeting GPC1

As previously mentioned, GPC1 is overexpressed in 98% of ESCC and its overexpression is associated with poor prognosis and chemoresistance [10]. The anti-GPC1 antibody, named 1-12, was isolated from chicken and recognizes both human and mouse GPC1 [65]. The chicken/mouse chimeric monoclonal antibody (mAb) with mouse IgG2a Fc domains induces 70% tumor growth inhibition in both ESCC xenograft models and patient-derived tumor (PDX) xenograft models in immunodeficient mice.

Antibody-drug-conjugates (ADC) are agents that consist of a highly cytotoxic small molecule linked covalently to a monoclonal antibody that recognizes a cell-surface antigen. Previous studies demonstrated regression of uterine cervical squamous cell carcinoma and uterine cervical adenocarcinoma after treatment with an ADC composed of an anti-GPC1 antibody (01a033) conjugated to monomethyl auristatin F (MMAF), an antitubulin agent that inhibits cell division [66].

Therapies Targeting GPC2

Recent studies show that GPC2 is overexpressed in neuroblastoma [4, 5]. Human single domain antibodies (sdAbs), e.g., LH7 against GPC2 were isolated from a phage-displayed human single domain antibody library. SdAbs could potentially target cryptic epitopes normally hidden for conventional antibodies. Other advantages of sdAbs include a small size, high solubility, thermal stability, refolding capacity, and good tissue penetration [67].

We developed two types of antibody therapeutics, immunotoxin and chimeric antigen receptor (CAR), to target GPC2 [5]. Immunotoxin is a chimeric protein composed of an antibody fragment fused to a toxin, such as the 38 kDa truncated fragment of *Pseudomonas* exotoxin (PE38). The immunotoxin LH7-PE38 inhibits neuroblastoma growth in mice [5]. CARs are synthetic receptors that target T cells to cell-surface antigens and augment T-cell function and persistence. CD19-targeted CAR T cells proved that engineered immune cells can serve as a powerful new class of cancer therapeutics [68, 69]; however, selecting appropriate targets in solid tumors is challenging. The tumor-specific expression of GPC2 makes it an attractive target for CAR T-cell therapy. In a recent study, we have showed that CAR T cells targeting GPC2 eliminated tumors in a disseminated neuroblastoma mouse model [5].

In addition, a GPC2-targeted ADC (D3-GPC2-PBD) was developed by conjugating an anti-GPC2 antibody (D3) with pyrrolobenzodiazepine (PBD) dimers [4]. D3-GPC2-PBD is efficacious in a neuroblastoma PDX model. Overall, GPC2 is a promising immunotherapeutic target for the treatment of neuroblastoma [4, 5].

Therapies Targeting GPC3

GPC3 is the most studied target, especially in HCC. Various therapeutic strategies were developed to target GPC3. Peptide vaccines of GPC3 have been extensively studied in recent years. In a Phase II clinical trial, major histocompatibility complex (MHC) class I restricted GPC3 peptides were given to HCC patients after surgery [70]. The GPC3 peptide vaccine improved the 1-year recurrence-free rate in patients with GPC3-expressing tumors. Interestingly, these MHC class I restricted peptides induce CD4⁺ T cell responses in nearly two thirds of patients resulting in prolonged 3-year and 5-year survival. Given the importance of CD4⁺ T cell induction, another study developed MHC class II restricted GPC3-derived long peptides (GPC3-LPs) to bind antigen-presenting cells (APC) and induced peptide-specific CD4⁺ T cell responses from most healthy donors [71]. Additionally, liposome-coupled GPC3-derived peptide (pGPC3-liposome) was investigated for its antitumor potential [72]. Peptide-specific CD8⁺ T cells (often called cytotoxic T lymphocytes or CTLs) were induced by pGPC3-liposome vaccine. The pGPC3-liposome

vaccine inhibits GPC3-expressing tumor growth. Furthermore, infusion of GPC3-coupled lymphocytes induces robust GPC3-specific antibody and T cell responses, and effectively inhibits proliferation of established HCC tumors by 70% in mice [73].

Several therapeutic anti-GPC3 antibodies have been developed as well. GC33 (mouse mAb) [74] and hYP7 (humanized mouse mAb) [75, 76] recognize the C-lobe of GPC3, and inhibit HCC tumor growth in animals by nearly 50% [74, 77]. Human single domain antibody HN3 targeting the N-lobe of GPC3 [6], and human antibody HS20 targeting the HS chains were also isolated [1, 6]. HN3 and HS20 inhibit HCC tumor growth in mice to approximately 80% and 67%, respectively. The GC33 antibody was well tolerated in a Phase I clinical trial in HCC patients [78], but did not show significant clinical benefit in a randomized Phase II clinical trial [79], suggesting that anti-GPC3 antibodies alone may have limited therapeutic effects in patients.

GPC3-targeted immunotoxins have also been evaluated in preclinical studies. HN3-PE38 immunotoxin regress HCC xenograft tumors in mice at 0.6 mg/kg dosage [49]. To reduce side effects and immunogenicity, great efforts have then been made to generate various forms of immunotoxins [80–82]. HN3-mPE24 is well tolerated in mice even at a dosage of 5 mg/kg and it induces significant tumor regression and increases survival in mice bearing HCC xenografts [80].

Photoimmunotherapy (PIT) employs a mAb-phototoxic phthalocyanine dye, IR700 conjugate, that is activated by near infrared (NIR) light and causes necrotic cell death. A recent study showed that PIT with IR700-YP7 and IR700-HN3 suppressed HCC tumor growth in mice by 40% [77, 83]. Although PIT is less effective than immunotoxins in mouse models, it uses a different mechanism of action and enables direct visualization of treatment.

Moreover, approaches that redirect T cells, including bispecific antibodies and CAR-T cell therapies, have been extensively explored. Bispecific antibodies can redirect T cells to tumor cells by engaging CD3 in the T cell and antigen in the tumor cell. Anti-GPC3/CD3 bispecific T cell-redirecting antibody (ERY974) regress GPC3-expressing solid tumors in mice [84], and is currently in Phase I clinical trial (NCT02748837ⁱ). GPC3-targeted CAR T cells have been shown to eliminate GPC3-expressing LSCC [85] and HCC cells [86]. Given the reported promising findings, several clinical trials using GPC3-targeted CAR T cells are underway (NCT02723942ⁱⁱ, NCT02395250ⁱⁱⁱ, NCT03198546^{iv}, NCT02876978^v).

In addition, GPC3-targeted CAR natural killer (NK) cells were generated by expressing a GPC3-specific CAR in NK cells [70]. Unlike CAR T cells, mature CAR NK cells have short lifespans and are expected to exhaust shortly after tumor lysis, with a turnover time of ~1–2 weeks [87]. Mature CAR NK cell grafting also has little risk of graft-versus-host disease (GvHD) as compared to CAR T cells [87, 88]. NK-92 is a highly cytotoxic NK cell line and can be continuously and homogeneously expanded; therefore, it has the potential to be

ⁱ<https://clinicaltrials.gov/ct2/show/NCT02748837>

ⁱⁱ<https://clinicaltrials.gov/ct2/show/NCT02723942>

ⁱⁱⁱ<https://clinicaltrials.gov/ct2/show/NCT02395250>

^{iv}<https://clinicaltrials.gov/ct2/show/NCT03198546>

^v<https://clinicaltrials.gov/ct2/show/NCT02876978>

developed into off-the-shelf products. In addition, NK-92 infusion was safe in clinical testing [89]. GPC3-targeted CAR NK cells inhibit 90% of GPC3-expressing HCC xenograft growth [90]. Primary NK cells overexpressing anti-GPC3 CAR are also cytotoxic to GPC3-expressing cells.

Lastly, a group of researchers generated a T cell receptor (TCR) targeting GPC3 [91]. They identified a dominant TCR (P1-1) that specifically bound a GPC3 peptide on HLA-A2. P1-1 expressing T cells killed HCC *in vitro*; however, they only delayed the growth of HCC xenograft tumors in mice [91].

Overall, the emerging new options of glypican-targeted therapies are summarized in Figure 3, and the ongoing preclinical and clinical developments are listed in Table 1 and 2, demonstrating the breath of scientific interest surrounding glypicans as therapeutic targets in cancer therapy.

Concluding Remarks

We have limited knowledge about the safety and efficacy of targeting tumor antigens to treat cancer, specifically in solid tumors. The potential damage in healthy tissues expressing targetable tumor antigens is of great concern. Thus, more work to determine the safety and efficacy profiles of such therapies is critical. Structural and functional analysis of glypicans and their role in pathobiological processes, particularly cancer development, provide an opportunity to identify a group of new tumor antigens in liver, pancreatic and childhood cancers. Building on recent advances in the development of GPC2 and GPC3-specific antibodies, immunotherapy could make a significant contribution in the field of glypican-targeted anticancer therapeutics. Continued efforts are underway to overcome critical barriers in developing successful T-cell immunotherapies for solid tumor (see Outstanding Questions). Additionally, the potential of newly developed single domain antibodies offer improved tissue penetration, buried functional epitope, and expression capacity affording advances in the field. Despite challenges, ongoing preclinical and clinical studies will establish and validate glypicans as new therapeutic targets for treating cancer.

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Highlights

- Selection of specific tumor antigens for cancer therapy remains a major challenge. Glypicans are expressed in cancers and embryonic tissues during development; however, their expressions are strictly suppressed in most adult normal tissues, which make glypicans attractive tumor-specific antigens.
- The most significant structural characteristic of glypicans is their various numbers of heparan sulfate chains close to the cell membrane. This unique structure endows glypicans the capability to rescue and sequester different growth factors, morphogens, chemokines and cytokines in physiological conditions. In this sense, glypicans act as essential co-receptors and trigger the activation of Wnt, Hh, FGF and many other signaling pathways.
- Unlike conventional full-size antibodies, single domain antibodies can bind to cavities on antigens, e.g., the buried active sites of enzymes. Together with other advantages including their small size, thermal stability, and good tissue penetration, single domain antibodies may become a new direction for next-generation antibody-based therapies.
- Glypican-targeted antibody-based therapies including: vaccine, immunotoxin, bispecific antibody and engineered T-cell therapy, are being intensively investigated in many preclinical and clinical studies. Efforts are now aimed at overcoming immunosuppressive microenvironment, improving T cell infiltration and persistence by developing new strategies and combination of existing therapies.

Outstanding Questions

- How can high-throughput sequencing, such as single-cell sequencing, be utilized to provide a better understanding of heterogeneity of glypicans expression and identify new mutations/isoforms of glypicans in patients for precision medicine?
- Can we develop biomarkers that can be used to identify patients who are the most likely to benefit from glypican-targeted therapy?
- Which combination treatments are most likely to act synergistically with therapies targeting tumor-specific glypicans?
- How can we better mobilize the immune system against glypicans to penetrate the highly immunosuppressive tumor microenvironment and eradicate solid tumors?

Text Box 1-**Evolution of Glypicans**

Glypicans are highly conserved during animal evolution. Here, we summarize the glypicans in various major species (Figure I, Text Box 1). The human genome has six glypican family members (GPC1–6) that can be grouped into two subfamilies: GPC1/2/4/6 and GPC3/5 that share approximately 25% amino acid identity [57]. Within the first subfamily, GPC4 and GPC6 are closely related, while GPC1 and GPC2 form a more divergent clade (Figure I, Text Box 1). Phylogenetic comparison reveals that all mouse, frog and fish proteins cluster with human orthologs

Of additional interest, invertebrate genomes typically contain two glypicans, where many vertebrates have six (or more) glypican genes, likely due to single gene and whole genome duplications. For example, there are two glypicans—Dally and Dally-like protein (Dlp) in *Drosophila melanogaster*. Dally is an ortholog of the human GPC3/5 subfamily, and Dlp is an ortholog of the GPC1/2/4/6 subfamily.

Text Box 2-**Structure of Glypicans**

All glypicans share an N-terminal secretory signal peptide, 14 evolutionarily conserved cysteine residues, an HS attachment domain near the C terminus, and a hydrophobic domain near the C terminus for the addition of the GPI anchor. Most glypicans are subjected to proteolytic cleavage *in vitro* and *in vivo*. The 30–40 kDa cleavage product generated from the N-terminus of the protein core remains attached to its C-terminal half by one or more disulfide bonds [92].

The core protein structures of Dlp and GPC1 have been previously reported [93, 94]. Despite only 25% of sequence identity, both proteins show cylindrical-like structures that are conserved between *Drosophila melanogaster* and humans (Figure II, Text Box 2). The N-, M-, and C-lobes are named according to their relative spatial position in the protein (Figure II, Text Box 2). Out of the 14 evolutionarily conserved cysteine residues, 12 are located in the N-lobe. The N-lobe is followed by a middle segment named M-lobe, which is stabilized by two conserved hydrophobic centers that connect the α -helices and also contains the C terminus of the protein. The C-lobe is the last part of the structure that contains a long loop that is processed by furin proteases in many of the glypican core proteins.

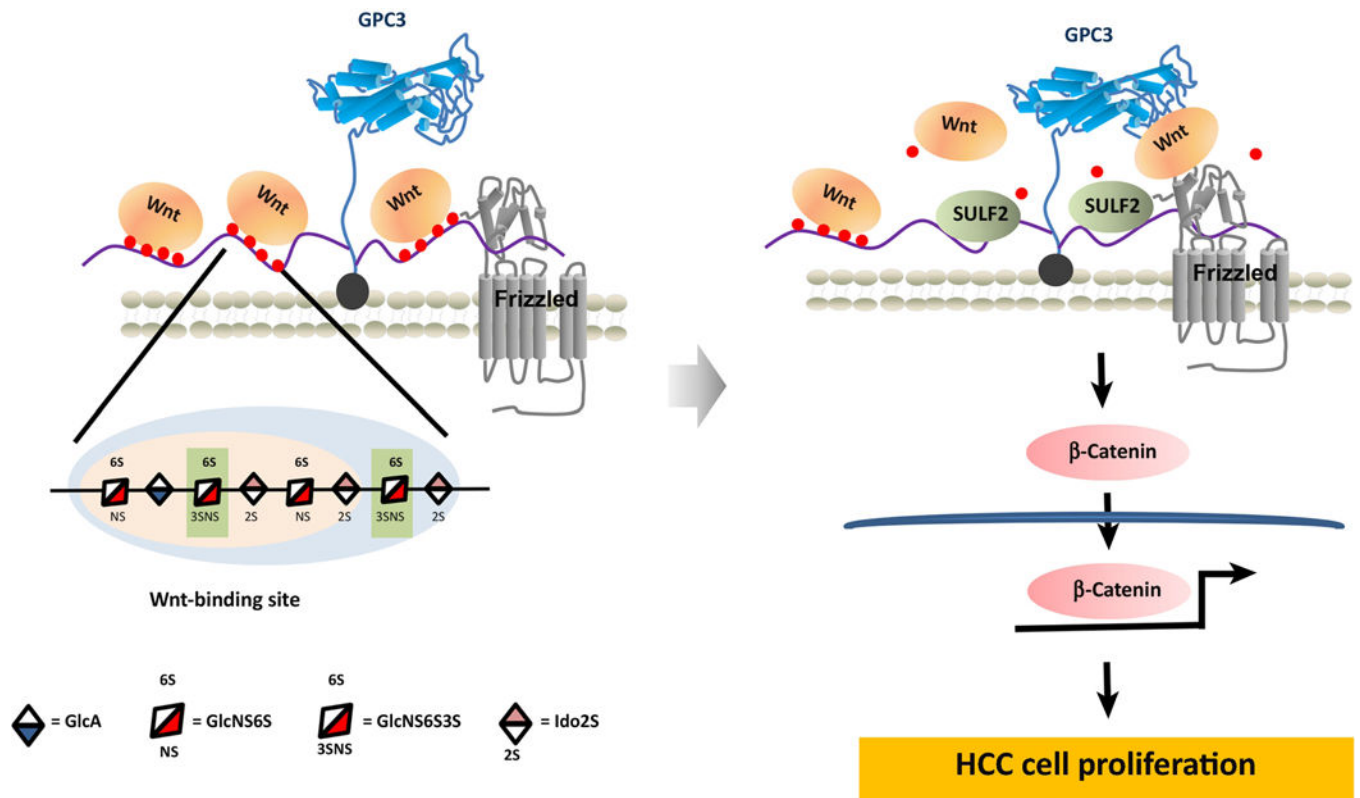


Figure 1. GPC3 Promotes Wnt Signaling through the Core Protein and HS Chains.

GPC3 interacts with frizzled through HS chains. Meanwhile, the HS chains also contain Wnt specific binding motif which needs 6-O-sulfation with 4 disaccharides in length (the light blue region). When an additional 3-O-sulfation presents, the region that recognized by Wnt could as short as 3 disaccharides in length (the light apricot region). GPC3 rescues circulating Wnt and acts as a Wnt storage site on cell surface through the HS chains. When SULF2 reduces the modification of the 6-O-sulfation on HS chains, the stored Wnt is released and forms a tripartite complex with glypican-3 core protein and frizzled, therefore activates canonical Wnt signaling, and promotes HCC cell proliferation.

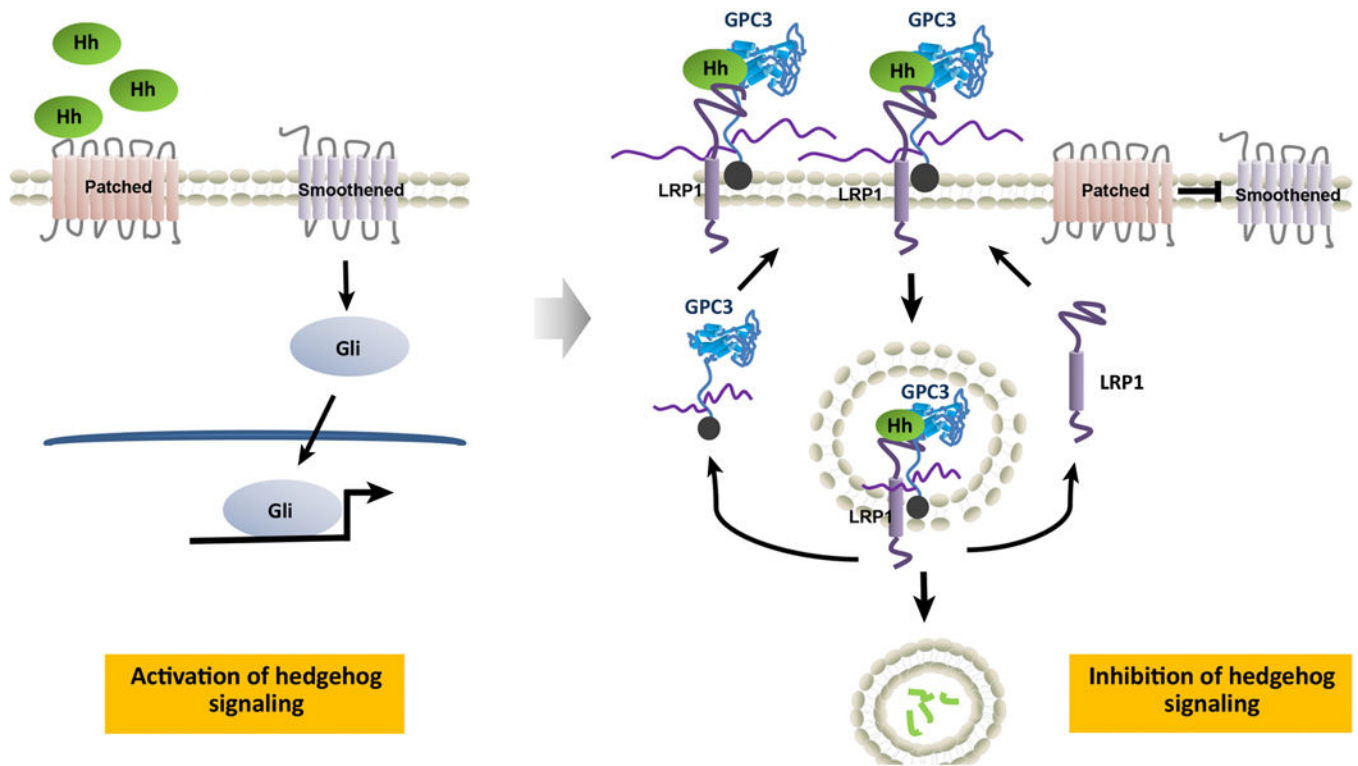


Figure 2. GPC3 Inhibits Hedgehog Signaling by Competing with Patched for Hh Binding. GPC3 binds to Shh and Ihh with high affinity. LRP1 promotes glypican-3/Hh complex internalization in a HS dependent way. The internalized Hh is transported to lysosome for degradation. Therefore, patched without Hh binding inhibits Smoothened activation and blocks Hedgehog signaling.

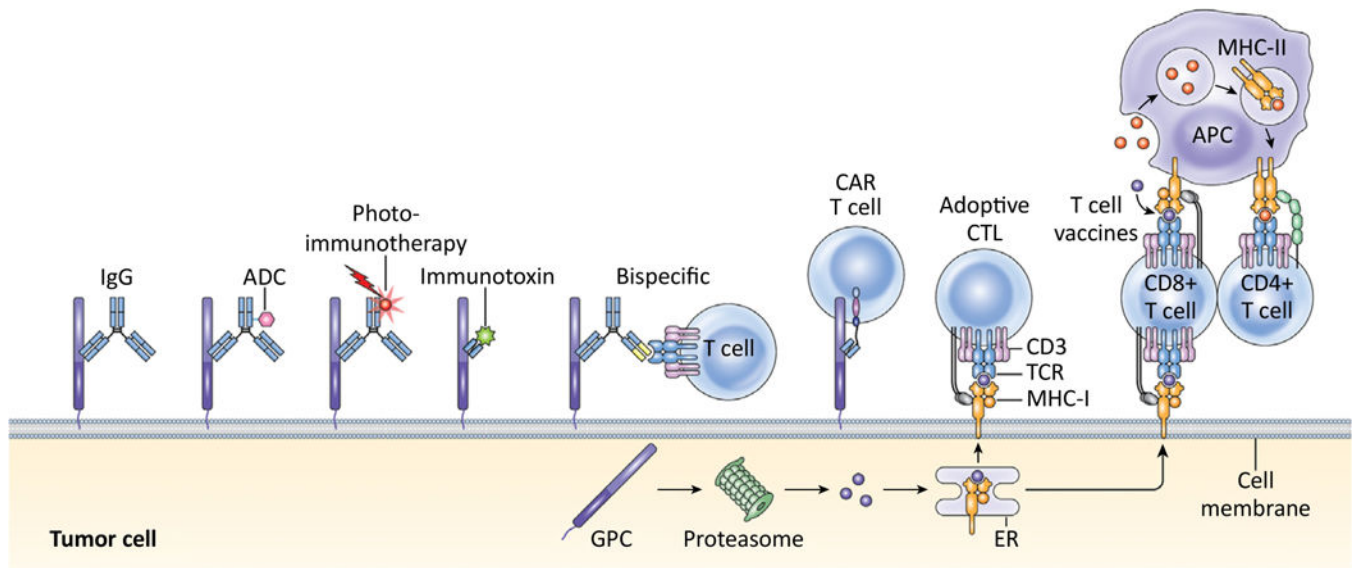


Figure 3. Antibody-based Therapies Targeting Glypicans in Cancer.

ADC: antibody- drug-conjugate; CAR: chimeric antigen receptor; CTL: cytotoxic T lymphocyte; TCR: T- cell receptor; MHC: major histocompatibility complex; APC: antigen-presenting cell; GPC: glypican; ER: endoplasmic reticulum (adapted with permission from [96]).

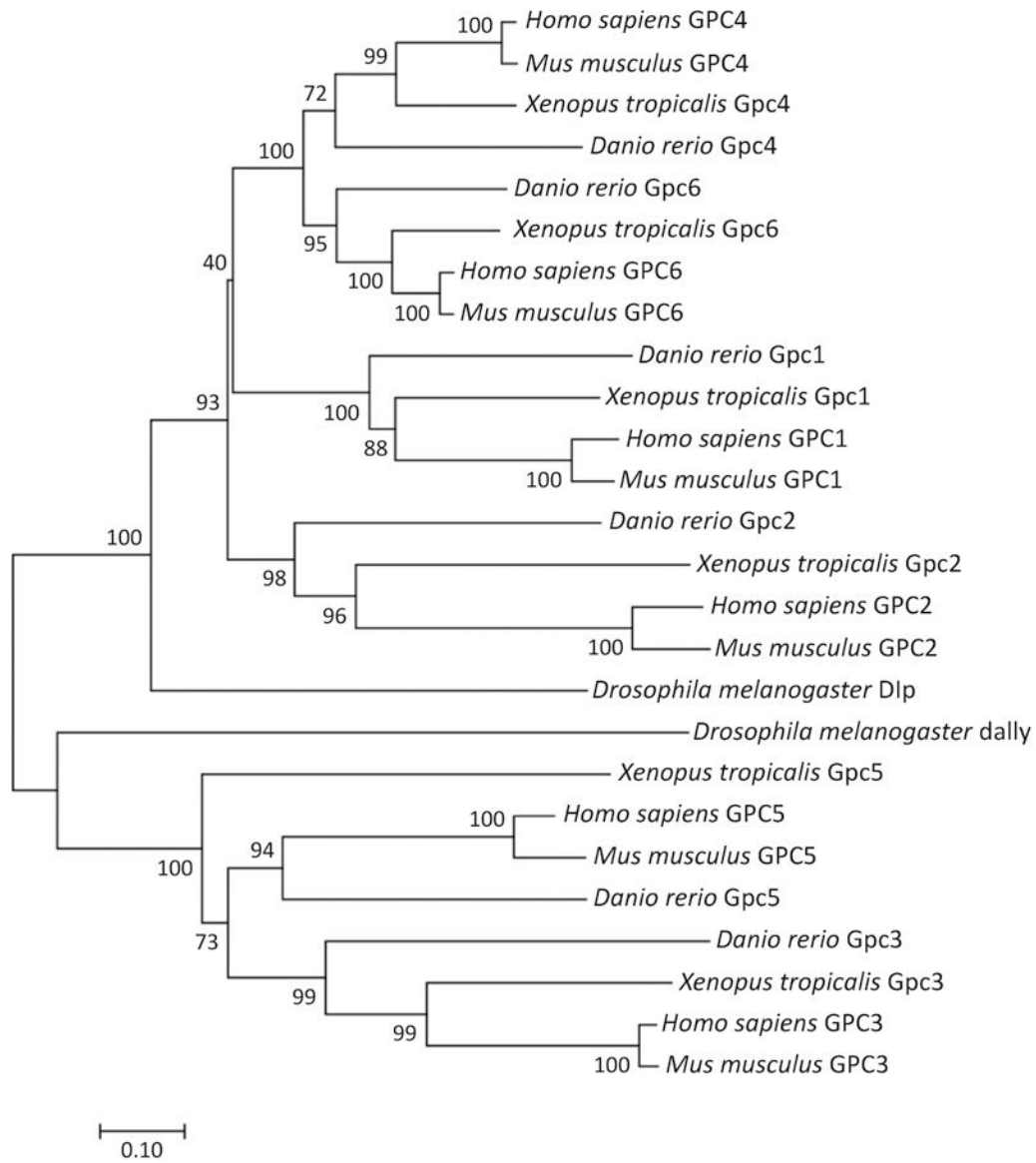


Figure I, Text Box 1. Glypicans Are Highly Conserved in Animals.

Phylogenetic comparison was carried out for glypicans protein sequences from *Homo sapiens*, *Mus musculus*, *Xenopus tropicalis*, *Danio rerio* and *Drosophila melanogaster*. The human genome has six glypican family members that can be grouped into two subfamilies: GPC1/2/4/6 and GPC3/5. Phylogenetic comparison reveals that all mouse, frog and fish proteins cluster with human orthologs. There are two glypicans–Dally and Dally-like protein (Dlp) in *Drosophila melanogaster*. Dally is an ortholog of the human GPC3/5 subfamily, and Dlp is an ortholog of the GPC1/2/4/6 subfamily. The evolutionary tree was constructed using Neighbor-Joining method in MEGA7 software using the Poisson model and partially deleted dataset. 1000 bootstrap replications were used as a test of phylogeny and the values are indicated next to the branch. Branch length corresponds to evolutionary distances that denote the number of amino acid substitutions per site. Scale bar: 0.1.

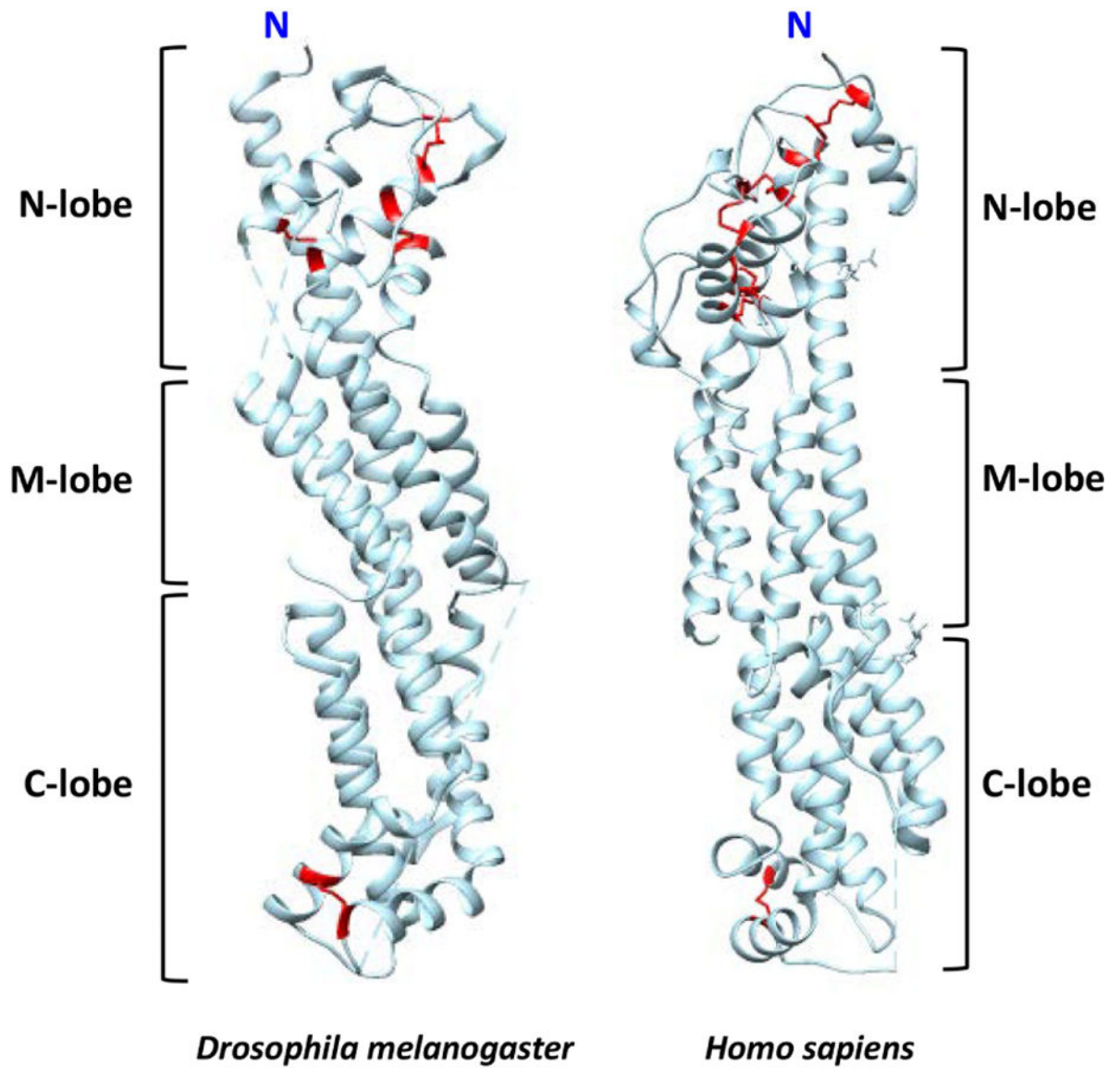


Figure II, Text Box 2. Structure of Dlp in *Drosophila melanogaster* and GPC1 in Humans. The termini are labeled, and the N, M, and C lobes are indicated. The N-, M-, and C-lobes are named according to their relative spatial position in the protein. The disulfide bonds are indicated (red). Both proteins show cylindrical-like structures that are conserved between *Drosophila melanogaster* and humans.

Table 1.

Cancer Therapies Targeting Glypicans in Preclinical Development

Target	Tumor type	Antibody	Format	Efficacy in animals	Reference
GPC1	ESCC	1-12	IgG	Tumor growth inhibition	[65]
	Cervical cancer	c01a033	ADC	Tumor regression	[66]
GPC2	Neuroblastoma	LH7	Immunotoxin	Tumor growth inhibition	[5]
			CAR T	Tumor regression	
	D3	ADC	Tumor regression	[4]	
GPC3	HCC	NA	LC/GPC3 ⁺	Tumor growth inhibition	[73]
		NA	Vaccine	Tumor growth inhibition	[72]
		HN3	IgG1	Tumor growth inhibition	[6]
		HS20	IgG1	Tumor growth inhibition	[1, 95]
		hYP7	IgG1	Tumor growth inhibition	[76]
		HN3	Immunotoxin	Tumor regression	[49, 80]
		YP7	Immunotoxin	Tumor growth inhibition	[49]
		GC33	CAR T	Tumor regression	[86]
		ERY974	Bispecific antibody	Tumor regression	[84]
		hu9F2	CAR NK	Tumor growth inhibition	[90]
		NA	TCR	Tumor growth inhibition	[91]
	GPC3-positive tumor cells	YP7	PIT	Tumor growth inhibition	[77, 83]
		HN3	PIT	Tumor growth inhibition	[83]
LSCC	GC33	CAR T	Tumor regression	[85]	

Table 2.

Clinical Development of GPC3-targeted Therapies

Tumor type	Antibody	Format	Phase	Status as of June 2018	Results	Reference
HCC	NA	Vaccine	II	Completed	Lowered the 1-year recurrence-free rate	[70]
HCC	GC33	IgG1	II	Completed	No clinical benefit	[78, 79]
Solid tumors	ERY974	Bispecific antibody	I	Recruiting	N/A	NCT02748837 ⁱ
HCC	GC33	CAR T	I	Ongoing but not recruiting	N/A	NCT02723942 ⁱⁱ
LSCC	GC33	CAR T	I	Recruiting	N/A	NCT02876978 ^v

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