

Review

Roles of Glypican-3 through IGF axis and Wnt in Hepatocellular Carcinomas

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Abstract:

Glypican-3 (GPC3) is overexpressed in more than 70% of hepatocellular carcinomas (HCCs) and also in Wilms' tumor, hepatoblastoma, melanoma, and neuroblastoma. Considerable evidences indicate that GPC3 alters the growth of cancer cells through a few different mechanisms. For example, GPC3 stimulates both canonical and noncanonical Wnt signaling in cancer cells and GPC3 inhibits Hedgehog signaling during development. We and others further demonstrate the role of IGF-1R in GPC3-mediated oncogenicity. In this review, we summarize the mechanisms underlying the consequences of GPC3 overexpression in HCC. We also review current GPC3-targeted therapeutic approaches for HCC.

Keywords: glypican-3 (GPC3); insulin-like growth factor 1 receptor (IGF-1R); hepatocellular carcinoma (HCC).

Introduction

The role of Glypican-3 (GPC3) in cancers has been confusing until recently. GPC3 was reported to display loss-of-function mutations in patients with the Simpson-Golabi-Behmel Syndrome (SGBS), an X-linked disorder characterized by pre- and post-natal overgrowth, a broad spectrum of visceral and skeletal abnormalities, and an increased risk for the development of embryonic tumors (1). Being aware that the insulin-like growth factors (IGFs) play a critical role in the regulation of body size, Pilia et al., proposed that GPC3 was a negative regulator of IGF-2, and that the loss of functional GPC3 in the SGBS patients generated hyperactivation of IGF signaling. These findings

together with cell line-specific promotion of apoptosis by rat GPC3 (2) suggest that GPC3 plays a negative role in cell proliferation and an apoptosis inducing role in specific tissues. Consistent with this idea, GPC3 is frequently silenced in ovarian cancer, mesotheliomas and breast cancer cell lines and ectopic expression of GPC3 inhibited the growth of these cells (3-5).

In contrast, GPC3 is frequently up regulated in hepatocellular carcinoma (HCC) (6, 7), neuroblastoma and Wilms' tumor (8), hepatoblastoma (9) and melanoma (10). We and Filmus et al., found that Glypican-3 promotes the growth of HCC (11, 12). Therefore, GPC3 induces apoptosis or promoting cell growth in a cell line

specific manner. This review is about the current knowledge on the structure and function of GPC3, the involvement of GPC3 in human pathologies, the different hypotheses with regard to the molecular basis of GPC3 function, and the improved therapies for HCCs in which GPC3 and these molecules are implicated.

Glypican-3 (GPC3) is a member of the GPC family

Glypican-3 (GPC3) is a glycosyl-phosphatidylinositol (GPI)-anchor heparan sulfate proteoglycan (HSPG) (13), and a member of the glypican family. Glypicans (GPCs) are heparan sulfate proteoglycans that are bound to the external surface of the plasma membrane by a GPI linkage (14). There are six glypican family members in the human genome (GPC1 to GPC6) (Fig. 1). Glypicans fall into two broad subfamilies: glypicans 1/2/4/6 and glypicans 3/5, with approximately 25% amino-acid identity between groups (15). Within the first subfamily, glypicans 4 and 6 are relatively closely related (64% identity) and glypicans 1 and 2 form a more divergent clade. A notable genomic feature of GPCs in the mouse and human genomes is the close linkage of genes that form two glypican clusters: glypicans 3/4 on the X chromosome (16, 17), and glypicans 5/6 on human chromosome 13 (mouse chromosome 14) (18, 19). Glypican proteins are between 555 and 580 amino acids in length, and encoded in eight to ten exons in human.

Structure of GPCs

Because of the lack of X-ray crystallography and other imaging data, knowledge of the three-dimensional structure of GPCs is limited. Furthermore, GPCs do not have domains that share significant homology with other characterized structures. However, the three-dimensional structure of GPCs appears to be highly conserved across the family, as the localization of 14 cysteine residues is preserved in all family members (20) and called cysteine-rich domain (CRD); whether this has functional implication remains unknown. Another noteworthy structural feature shared by all GPCs is the insertion sites for the heparan sulfate (HS) chains, a type of glycosaminoglycan (GAG) (Figure

1), which are located close to the carboxyl terminus (21, 22). This places the HS chains close to the cell surface, suggesting that these chains can mediate the interaction of GPCs with other cell-surface molecules, including growth factor receptors (13).

Most GPCs, including those of *Drosophila* (23), are processed by endoproteolytic cleavage by a furin-like convertase (22) (Figure 1). The cleavage site is located at the carboxy-terminal end of the CRD, and the cleavage generates two subunits that remain attached to each other by one or more disulfide bonds (22). After proteolysis by the convertases, the mature proteins/peptides are usually subjected to several other modifications necessary to achieve full bioactivity (24). Therefore, the cleavage of GPCs by furin-like convertase may be vital for their functions. GPC3 contains a proline-rich region located at residues 26–31, which is crucial for its association with insulin-like growth factor (IGF)-2 and IGF-1R and its internalization after IGF-1 treatment (12, 25).

Subcellular localization and function of GPCs

As expected for proteins carrying GPI anchors, GPCs are mostly found at the cell membrane (13, 14, 20, 26). In polarized cells, GPI-anchored proteins are usually located at the apical membrane and in the lipid rafts (27). Lipid rafts are cell-membrane subdomains that are glycolipid-enriched and detergent resistant (28-30). GPCs function as co-receptors for heparin-binding proteins, including growth factors, extracellular matrix molecules, and cell–cell adhesion molecules (31, 32). The binding of co-receptors may facilitate interactions between the heparin-binding factors and their respective receptors (3, 33). Cytoplasmic translocation of GPC3 has also been observed (14, 25).

GPCs can also be shed into the extracellular environment. This shedding is generated, at least in part, by Notum, an extracellular lipase that releases GPCs by cleaving the GPI anchor (34, 35). GPCs have been found in lipophorins, the *Drosophila* lipoproteins on the plasma membrane (23). GPCs can recruit lipophorin to disc tissue and remain associated with lipophorins after they are released from the

Table 1. Summary of GPC3 associated pathways in cells or mice

Cell types or mice	Pathways	Results	Ref.
HCC cells	IGF axis	Promote cell growth	(12, 25)
HCC cells	Canonical Wnt	Promote cell growth	(11)
Breast cancer cells (MCF), Mesothelial cells (II14)	Noncanonical Wnt	Apoptosis	(22, 49)
GPC3 deficient mice	Hedgehog	Gli-1↑,Ptc1↑	(50)
GPC3 in mice	BMP2 and BMP4	-	(51, 52)

membrane by anchor cleavage (23). Lipophorin is important for long-range, but not short-range, signaling activity of Wg and Hh (23, 36).

Not only GPC3 but also other GPCs are associated with cancer. GPC1 is strongly expressed in human pancreatic cancers, both in cancer cells and adjacent fibroblasts, whereas expression of GPC1 is low in normal pancreatic cells and in chronic pancreatitis (37). GPC1 can facilitate the interaction between FGF2 receptor and stimulate FGF2 signaling (38). GPC2 is highly expressed in neuroblastoma and required for neuroblastoma proliferation (39). GPC4 is specifically required to maintain the self-renewal potential of mouse embryonic stem cells (ESCs) *in vitro* by modulating Wnt/ β -catenin signaling and to fine tune cell lineage commitment (40). GPC5 is a novel epigenetically silenced tumor suppressor, which inhibits tumor growth by suppressing Wnt/ β -catenin signaling in lung adenocarcinoma (41) and prostatic cancer (42). GPC6 is overexpressed in early stage ovarian cancer and correlated with increased patient survival (43).

GPC3 overexpression in HCC

GPC3, preferentially expressed in HCC (previous named as MXR7) (6), was overexpressed in primary and recurrent HCCs. GPC3 overexpression has been correlated with high α -fetoprotein (AFP) levels, high tumor grade, high tumor aggressiveness (6), later tumor stage, presence of vascular invasion, and shortened overall survival and disease-free survival in HCC individuals (44). These findings suggested that GPC3 may play a role in cancer invasion and progression and may be

related to poor prognosis of HCC. GPC3 mRNA expression in HCC occurred more frequently in female than in male patients (95% versus 67%, $P = 0.0227$), consistent with the localization of GPC3 on chromosome Xq26.1. These findings have been confirmed by other investigators (7, 45, 46). Overexpression of GPC3 mRNA was found in 75% of HCCs but not in focal nodular hyperplasia and cirrhotic liver (7). Capurro et al. (45) found that GPC3 was undetectable in all healthy individuals, whereas 53% of HCC patients had significantly elevated levels of serum GPC3 with values ranging from 150 to 3000 ng/mL, but was detected in only one of the patients with hepatitis and cirrhosis (117 ng/mL). These findings suggest that GPC3 is a useful serological marker for HCC (7, 45). However, a meta-analysis of ten studies showed a pooled sensitivity for AFP and GPC3 of 51.9 % and 59.2 %, respectively, while the pooled specificity for AFP and GPC3 is 94 % and 84.8 % (47). The diagnostic sensitivity for HCC increased to 72.8% when GPC3 was combined with AFP. GPC3 was not a promising serum maker for the diagnosis of HCC alone, but it could be complementary to AFP to elevate the sensitivity of HCC diagnosis (48).

Signaling pathways activated by GPC3 overexpression in HCC

The Wingless/Wnt, Hedgehog, Dpp/Bmp and IGF signaling pathways have all been associated with GPC3 overexpression in HCC (Figure 2) (Table 1) (1, 49-51, 53).

Two pathways are associated with Wnt signaling: the canonical and noncanonical pathways (54). Capurro et al. showed that the

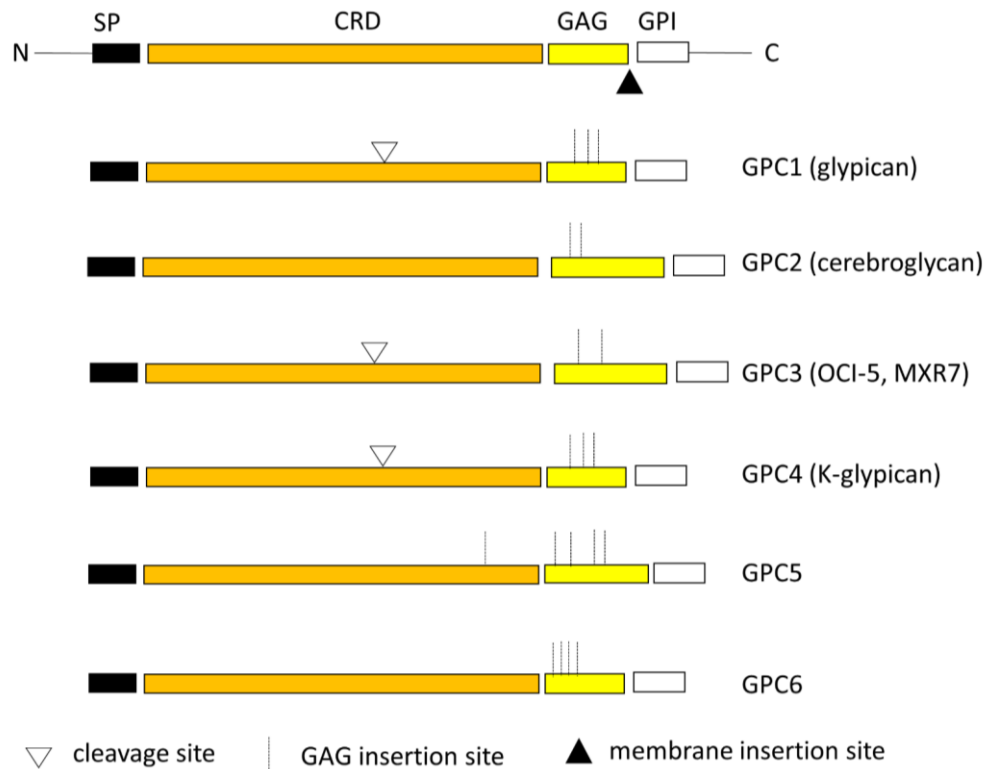


Figure 1. Schematic representation of GPCs. The GPCs domains are depicted as boxes. Orange boxes denote CRD which are conserved in all GPCs. GAG chains can be found along the whole core protein, and appear to be restricted to the last 50 amino acids of the mature core protein, just upstream of the membrane insertion (black triangle). There is an internal proteolytic cleavage site in three glypican family members (open triangle). SP, signal peptide for membrane translocation; CRD, cysteine-rich domain; GAG, glycosaminoglycan; GPI, glycosyl-phosphatidylinositol.

nonglycanated core protein of the internal HA-tagged OCI-5/GPC3 could form complexes with Wnts and stimulated canonical Wnt activity by facilitating the interaction with its signaling receptors, leading to the stabilization of β -catenin in HCC cells (11) (Fig. 2a). De Cat et al. found that an internal HA-tagged GPC3 could inhibit Wnt signaling in MCF7 cells and could induce apoptosis by activating c-Jun NH2-terminal protein kinase (JNK) (22) (Fig. 2b). Song et al. found that internal HA-tagged OCI-5/GPC3 could form complex with Wnt 5a (49) and FGF2 (55), but not IGF-2 (55), and could induce apoptosis by activation of the noncanonical Wnt, inhibition of canonical Wnt and reduction of cytosol β -catenin in mesothelial cell

line II14 (49) (Fig. 2b). These observations suggest that GPC3 modulates both the canonical and noncanonical Wnt pathways depending upon cell types. GPC3 can stimulate canonical Wnt signaling and promote HCC cell growth (11). By contrast, GPC3 can induce noncanonical Wnt signaling and then inhibit the canonical Wnt signaling pathway, causing cell apoptosis in breast cancer cells (22) and mesothelial cells (49). Hence, the different GPC3 functions may be due to differences in Wnt signaling pathway involved (11, 22, 49).

Although haven't been associated with HCC, Hedgehog and Bone morphogenetic proteins (BMPs) play roles in GPC3 function in development (Fig. 2c and 2d). Capurro et al. found that the

Table 2. Summary of GPC3 associated therapies

Material injected	Study design	Outcome	Ref
GPC3 peptide-specific cytotoxic T cells (CTL)	Subcutaneous inoculated GPC3 expressing colon26 cancer cells	Rejection of the tumor in vivo	(72)
Peptide vaccine	Patients with advanced HCC	Induced production of GPC3-peptide specific CTL and tumor necrosis	(73)
GC33 monoclonal Ab	Subcutaneous inoculated HCC cells	Ab-dependent cellular cytotoxicity anti-tumor activity	(74)
Anti-GPC3 Ab fused with Pseudomonas exotoxin	Liver tumor xenografted in mice	Tumor regression	(75)
AntiGPC3/CD3 bispecific Ab (ERY974)	Subcutaneous inoculated GPC3 expressing cell lines	Highly effective in killing GPC3 expressing tumors	(76, 77)
Soluble GPC3 lacking GPI anchor domain	Subcutaneous inoculated HCC cells	Inhibited HCC tumorigenicity	(78, 79)
GPC3-specific NK cells, NK-92/9.28.z	GPC3(+) HCC xenografts	Decreased tumor proliferation and increased tumor apoptosis	(80)
MicroRNA miR-4510	hepatoma cells	Induced apoptosis and blocked tumor growth in vivo	(81)

relative mRNA levels of two well-characterized Hh targets, Gli-1 and Ptc1, were significantly elevated in GPC3-deficient mouse embryos compared with their normal littermates (50). GPC3 interacted with Hedgehog but not its receptor, Patched, leading to Hedgehog endocytosis and degradation (50). BMPs were originally identified by an ability of demineralizing bone extract to induce endochondral osteogenesis *in vivo* in an extraskeletal site (56). Paine-Saunders et al. found that *Bmp4* expression in the developing limb was coincident with the *Gpc3* expression zone, in the necrotic zones and in the interdigital web mediating apoptotic events in the development of these structures (51). The offspring of GPC3-deficient animals mated with mice haploinsufficient for *Bmp4* displayed a high penetrance of postaxial polydactyly and a lack of rib malformations in either parent strain, suggesting a link between GPC3 and BMP4 function (51). BMP2 exerted a strong inhibitory effect on *Gpc3*-positive cells, whereas keratinocyte growth factor (KGF; also termed FGF7) stimulated

tubule formation by *Gpc3*-positive cells dose-dependently, suggesting that GPC3 modulates both inhibitory and stimulatory pathways, depending on the growth factors (52). Similar findings were found in rat glypican that it strongly inhibits the mitogenic response to keratinocyte growth factor (KGF) but enhances the response to acidic fibroblast growth factor (FGF) in keratinocytes (57). These findings demonstrate that glypican can either enhance or suppress cellular responsiveness for different growth factors. Thus, the dual function of GPC3 may be cell type specific or growth factor specific.

GPC3 and the IGF axis in HCC

The IGF axis is one of the most commonly deregulated signaling pathways that contribute to cancer development. Ample preclinical evidence indicates that all four components—ligands, receptors, substrates, and ligand-binding proteins [IGFBPs]—of the IGF axis are crucial in the carcinogenic and metastatic potential of HCC (58). Overexpression of IGF-2 has been estimated to

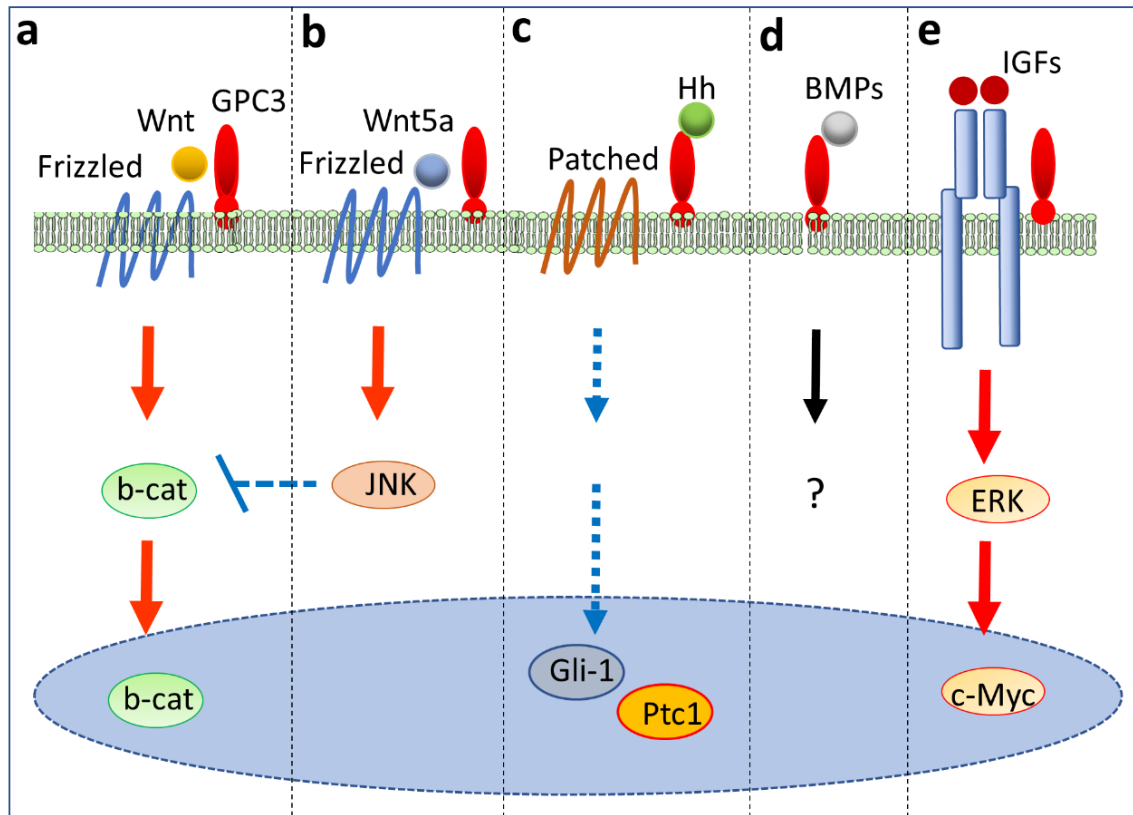


Figure 2. Pathways associated with GPC3. (a) GPC3 stimulates Wnt signaling by facilitating/stabilizing Wnt-Frizzled interaction in HCC. GPC3 stabilizes the binding of Wnt ligand to Frizzled and leads to stabilization of hypophosphorylated β -catenin. (b) GPC3 activates non-canonical pathway and leads to the inhibition of canonical Wnt signaling in II14, MCF7 and LM3 cells. GPC3 may form a complex with Wnt5a that enhances Wnt5a-dependent non-canonical signaling, activates JNK, then inhibits canonical signaling through a cross talk mechanism, and decreased cytosolic β -catenin. (c) GPC3 present at the cell membrane competes with Ptc for Hh binding and inactivates the Hh signaling in mouse embryos. As a consequence, there is a reduction in the amount of Hh targets, Gli-1 and Ptc1. (d) There are links between GPC3 and BMPs in mouse embryos, while the downstream signals are unclear. (e) Links between GPC3 and IGF receptor in HCC cells.

occur in 16%-40% of human HCC (59) and IGF-2 overexpression correlates with higher cell proliferation in both *in vivo* and *in vitro* models of HCC (60, 61). IGF-IR overactivation is one of the hallmarks of HCC and can be mediated by increased levels of IGF-IR protein and/or excess of IGF ligands (62). Healthy mature hepatocytes do not express IGF-IR, while, upregulation of IGF-IR occurs in the HCC samples of 30% of the patients (63).

GPC3 is involved in the pathogenesis of SGBS (1), which previously was often diagnosed as Beckwith–Wiedemann syndrome (64, 65), an

autosomal disorder linked to the short arm of chromosome 11 (66, 67). These two syndromes share many clinical features. Increased action of IGF-2 may cause the comparable tissue overgrowth seen in Beckwith–Wiedemann syndrome (68). GPC3 may interfere with IGF-2 signaling by sequestering or downregulating IGF-2 and inducing apoptosis in embryo development (1, 53). Using Western blot and ligand blotting experiments, Pilia et al. demonstrated that GPC3 formed a complex with IGF-2, thereby modulating IGF-2 action (1). GPC3 mutated in these prolines (P26-30A), when expressed in cells, could be

localized to the cell surface, but could not interact with either IGF-2 or IGF-1R or be internalized into cells after IGF-1 treatment (25).

GPC3 binds to IGF-2, activates IGF-1R, and then triggers a phosphorylation cascade including IGF-1R itself and ERK in HCC (Fig. 2e) (Table 1) (12). ERK mediates IGF-2-induced gene expression, cell invasion, and apoptosis protection (69, 70). ERK also contributes to multistep hepatocarcinogenesis (71). GPC3 decreases IGF-1-induced IGF-1R ubiquitination and degradation and increases c-Myc protein levels in HCC cells (25). GPC3 binds to Grb10, a mediator of ligand-induced receptor ubiquitination, and the overexpression of Grb10 blocks GPC3-enhanced IGF-1-induced ERK phosphorylation. More importantly, IGF-1R is essential for the activation of ERK and the increase of c-Myc by GPC3, as evidenced by the negative finding of ERK phosphorylation or c-Myc overexpression in IGF-1R negative R-cells expressed GPC3 (25). Therefore, GPC3 stimulated the phosphorylation of IGF-1R and the downstream signaling molecule extracellular signal regulated kinase (ERK) in an IGF-1 or IGF-2 dependent way. Moreover, GPC3 binds to and potentially sequesters Grb10, thereby blocking IGF-1R ubiquitination and degradation in the proteasome (25). The status of receptor internalization in the presence of GPC3 or the mechanism for the cytoplasmic translocation of GPC3 is currently not clear.

GPC3 as a target in therapy of HCC

GPC3 is frequently expressed in HCC (6) and can promote HCC growth (11). It is a membrane protein (13) and can be detected in the blood (45). Nakatsura et al. found that it was highly immunogenic in mice and could be an effective tumor antigen (72). Injection of the GPC3 peptide-specific cytotoxic T cell (CTL) into the subcutaneously inoculated colon26 cancer cells transfected with the mouse *GPC3* gene (C26/GPC3) led to the rejection of the tumor *in vivo*. The intravenous inoculation of these CTLs into sublethally irradiated mice markedly inhibited the growth of an established subcutaneous tumor (Table 2) (72). In the clinical trial of GPC3 peptide vaccine in patients with advanced hepatocellular

carcinoma, the vaccination induces production of GPC3-peptide specific CTLs and infiltration of GPC3-specific CTLs into the tumor is correlated with tumor necrosis (Table 2) (73). Therefore, GPC3 peptide vaccination represents a promising approach for the treatment of HCC. Moreover, Ishiguro et al. showed that the monoclonal antibody GC33 against the COOH-terminal region of GPC3 induced antibody-dependent cellular cytotoxicity and elicited antitumor activity in an antigen-dependent manner in human HCC cells (Table 2) (74). Therapeutically targeting GPC3 using a conformation-specific single-domain antibody in HCC has been reported (82). An anti-GPC3 antibody can be fused to a fragment of *Pseudomonas* exotoxin A (PE38) to create immunotoxins to induce the regression of liver tumor xenografts in mice (Table 2) (75). ERY974, a whole humanized immunoglobulin G-structured TRAB (T cell-redirecting antibody) which bispecifically binds to GPC3 and CD3, is highly effective in killing GPC3 expressing clinical tumors (Table 2) (76). Similar bispecific T cell engager (BiTE) targeting Glypican 3 (GPC3) and CD3 was also reported by Bi et al. (77). A soluble GPC3, lacking the GPI-anchoring domain, has also been shown to inhibit HCC cell growth, probably through competition with endogenous GPC3 for protein binding (78, 79). GPC3-specific chimeric antigen receptor (CAR)-modified natural killer (NK) cells, NK-92/9.28.z cell line, could induce significant *in vitro* cytotoxicity and cytokine production, decrease tumor proliferation, and increase tumor apoptosis in the GPC3⁺ HCC xenografts (80). MicroRNA miR-4510 induced apoptosis of hepatoma cells and blocked tumor growth *in vivo* through direct targeting of GPC3 mRNA and inactivation of Wnt/ β -catenin transcriptional activity and signaling pathway. miR-4510 also up-regulated the expression of several tumor suppressor genes while reduced the expression of other pro-oncogenes. Therefore microRNA has potential in HCC therapy (81). In the present study, the mechanisms of GPC3-mediated enhancement of IGF-1R are further elucidated and may be targeted to treat HCC and to improve the outcome of patients in the future.

Conclusion

GPC3 is overexpressed in most of the HCC tumors and several other kinds of tumors, and was also used as a useful marker for HCC diagnosis and prognosis. It is used as a potential target for developing therapeutic antibodies for HCC treatment. Several studies described the manipulation of GPC3 expression can be used to regulate Wnt and IGFs pathways in HCC. IGF-1R blockage has also been tested as a treatment for cancers, including HCC (83), but there were serious adverse events including dehydration, asthenia, and hyperglycemia (84, 85). In the future, the mechanisms of GPC3-mediated enhancement of IGF-1R will be further elucidated and these mechanisms may be targeted to treat HCC and to improve the outcome of patients. These discoveries therefore have repercussions that go beyond HCC, because the Wnt or IGFs pathway plays a critical role in various types of cancers.

Abbreviations lists

AFP, α -fetoprotein
 BiTE, bispecific T cell engager
 CAR, chimeric antigen receptor
 CRD, cysteine-rich domain
 CTL, cytotoxic T cell
 Dpp/Bmp, Decapentaplegic/Bone morphogenetic protein
 ERK, extracellular signal regulated kinase
 ESCs, embryonic stem cells
 FGF, fibroblast growth factor
 GAG, glycosaminoglycan
 GPC3, glypican-3
 GPI, glycosyl-phosphatidylinositol
 HCC, hepatocellular carcinoma
 HS, heparan sulfate
 IGF, insulin-like growth factor
 JNK, c-Jun NH₂-terminal protein kinase
 KGF, keratinocyte growth factor
 NK, natural killer
 SGBS, Simpson-Golabi-Behmel Syndrome
 TRAB, T cell-redirecting antibody

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