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REVIEW ARTICLE

Glypican-3: A promising biomarker for hepatocellular carcinoma diagnosis and treatment

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Abstract

Liver cancer is the second leading cause of cancer-related deaths, and hepatocellular carcinoma (HCC) is the most common type. Therefore, molecular targets are urgently required for the early detection of HCC and the development of novel therapeutic approaches. Glypican-3 (GPC3), an oncofetal proteoglycan anchored to the cell membrane, is normally detected in the fetal liver but not in the healthy adult liver. However, in HCC patients, GPC3 is overexpressed at both the gene and protein levels, and its expression predicts a poor prognosis. Mechanistic studies have revealed that GPC3 functions in HCC progression by binding to molecules such as Wnt signaling proteins and growth factors. Moreover, GPC3 has been used as a target for molecular imaging and therapeutic intervention in HCC. To date, GPC3-targeted magnetic resonance imaging, positron emission tomography, and near-infrared imaging have been investigated for early HCC detection, and various immunotherapeutic protocols targeting GPC3 have been developed, including the use of humanized anti-GPC3 cytotoxic antibodies, treatment with peptide/DNA vaccines, immunotoxin therapies, and genetic therapies. In this review, we summarize the current knowledge regarding the structure, function, and biology of GPC3 with a focus on its clinical potential as a diagnostic molecule and a therapeutic target in HCC immunotherapy.

KEYWORDS
glypican-3, hepatocellular carcinoma
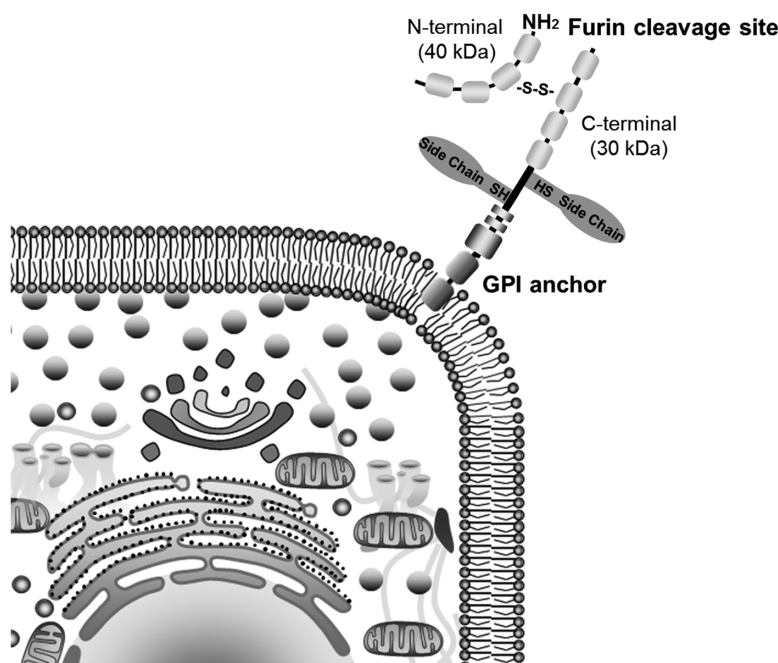
1 | INTRODUCTION

The latest global cancer statistics indicate that liver cancer ranks second among the causes of cancer-related deaths.[1] Hepatocellular carcinoma (HCC) is the most common type of liver cancer. Although surgery is the treatment of choice, only 5–10% of HCC patients are candidates for surgical resection.[2] Thus, specific HCC biomarkers and molecular targets hold considerable clinical significance for the early diagnosis and development of targeted therapies for patients with HCC.

Numerous proteins and receptors are overexpressed on HCC cell membrane, including glypican-3 (GPC3), asialoglycoprotein receptor, glycyrrhetinic acid receptor, CD71, homodimeric glycoprotein, somatostatin receptor, CD44, lysosomal-associated protein transmembrane-4β, and retinoic acid receptors.[3] Of these, GPC3 has attracted substantial attention because its expression is correlated with HCC tumorigenesis and prognosis.[4,5] Both basic and clinical research has indicated that GPC3 is not only a highly specific diagnostic biomarker, but also an immunotherapeutic target for HCC.[6,7]

GPC3 is a member of the glypican family and its expression is normally detected in placenta, numerous embryonic tissues, adult ovary, mammary gland, mesothelium, lung, and kidney.[8,9] In healthy adult liver, no GPC3 expression is detected. By contrast, GPC3 overexpression has been detected in HCC; GPC3 protein and gene expression levels in the serum and tumor tissue are higher in HCCs than in healthy or nonmalignant livers.[6] Furthermore, GPC3 expression discriminates alpha-fetal protein (AFP)-negative HCC from benign nodules, and thus GPC3 is regarded a more reliable marker than AFP in HCC diagnosis.[10–12] Here, we summarize the diagnostic, prognostic, and therapeutic value of GPC3 in HCC and discuss the mechanisms underlying GPC3 involvement in HCC progression in order to comprehensively review the role of GPC3 in HCC.

2 | STRUCTURE AND EXPRESSION OF GLYPICANS

GPC3 is a member of the heparan sulfate (HS) proteoglycan family, which comprises six subtypes (GPC1–6). All glypicans share similarities, including attachment to the cell membrane via a glycosylphosphatidylinositol (GPI) anchor, a conserved pattern of 14 cysteine residues, the insertion sites of the HS side chains localized to the last 50 amino acids in the C-terminus, etc.[13] All glypicans are highly expressed during embryonic development.[9] GPC1 expression is detected in embryonic bone marrow, muscle epidermis, and kidney.[9] GPC2 is mainly expressed in nervous system. GPC3 is observed in placenta and numerous embryonic tissues.[9] GPC4 is expressed in embryonic brain, kidney, and lung.[9] GPC5 expression is observed in embryonic brain, lung, liver, kidney, and limb.[9] GPC6 is highly expressed in many embryonic tissues, including liver and kidney.[9] The main function of glypicans is to regulate the developmental signaling pathways of Wnt, Hedgehog, bone morphogenetic proteins (BMP), and fibroblast growth factors (FGF).[14] In normal adult tissues, GPC1 and GPC4 expression is detected in most tissues, while GPC2 is not detected.[9] GPC3 is expressed in adult ovary, mammary gland, mesothelium, lung, and kidney.[9] GPC5 is mainly observed in brain, and GPC6 is detected in many tissues.[9] In human diseases, GPC1 exosomes have been investigated as a specific biomarker for the early diagnosis of pancreatic cancer in recent years.[15,16] Other GPC1-related diseases include breast cancer,[17] esophageal squamous cell carcinoma,[18] ameloblastoma,[19] prostate cancer,[20] neuroendocrine tumors,[21] etc. The association between GPC2 and human diseases has not been clarified to date. GPC3 functions as an oncofetal protein and is involved in different HCC signaling pathways, including Wnt.[22,23] Other GPC3-associated diseases include melanoma, ovarian cancer, squamous cell carcinoma of the lung, testicular nonseminomatous germ cell tumors, liposarcoma, and embryonal tumors such as neuroblastoma, and Wilm's tumor.[24–26] GPC4 is associated with body fat distribution, insulin signaling, and nonalcoholic fatty liver disease.[27–29] GPC5 acts as an oncogene in rhabdomyosarcoma[30,31] and a tumor suppressor in non-small cell lung cancer.[32,33] GPC6 is reported to be correlated with gastric adenocarcinoma, although this association remains to be validated.[34]
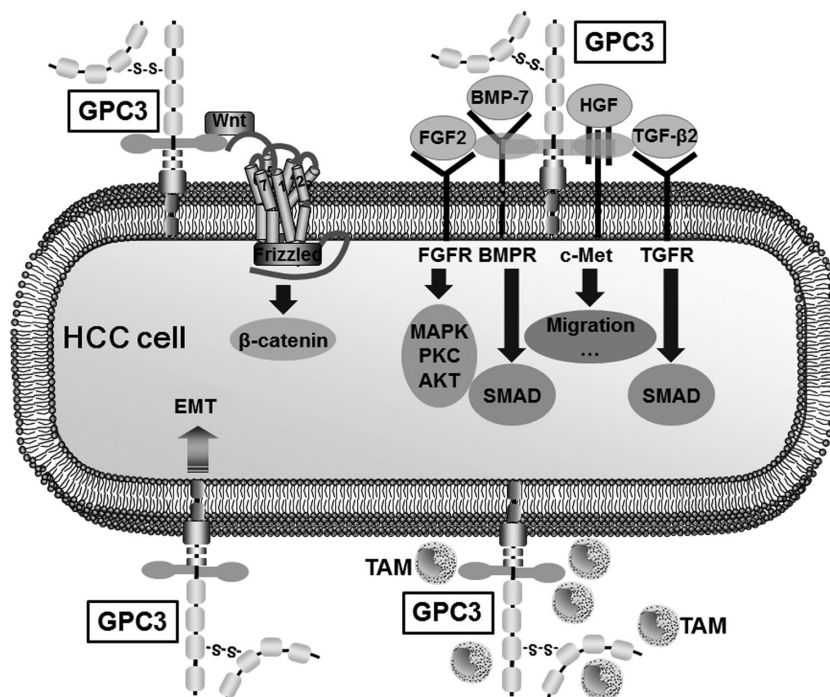
FIGURE 1 Structure of Glypican-3. Glypican-3 is a 70 kDa protein and can bind to the exocytoplasmic surface of the cell membrane through its GPI anchor. Glypican-3 is endoproteolytically cleaved by furin-like convertases between Arg358 and Ser359, which produces a 40 kDa N-terminal subunit and a 30 kDa C-terminal subunit harboring two HS side chains. These subunits remain linked to each other by one or more disulfide bonds. GPI, glycosylphosphatidylinositol; HS, heparan sulfate.

3 | STRUCTURE, EXPRESSION, AND FUNCTION OF GPC3

The human *GPC3* gene is located on the X chromosome (Xp26) and encodes a 70 kDa core protein containing 580 amino acids (Fig. 1).[22] GPC3 is endoproteolytically cleaved by furin-like convertases between Arg358 and Ser359, which produces a 40 kDa N-terminal subunit and a 30 kDa C-terminal subunit harboring two HS side chains.[22] These subunits remain linked to each other by one or more disulfide bonds.[35] Convertase processing is essential for GPC3 modulation of Wnt signaling, cell apoptosis, and gastrulation movements in zebrafish, while it is not required for GPC3-induced stimulation of HCC growth.[22] The binding of GPC3 to the exocytoplasmic surface is important for GPC3 modulation of cell growth and Wnt signaling.[23] In HCC patients, some forms of secreted GPC3 have been detected and reasons for the shedding of GPC3 have been investigated. One proposed mechanism is that an extracellular lipase named Notum releases GPC3 from the cell surface at the GPI anchor.[36] However, a recent study revealed that Notum does not cleave GPC3. Instead, it acts as a Wnt deacetylase.[37] Therefore, further mechanistic studies are needed regarding the cleavage of GPC3 from the cell membrane.

GPC3 is ubiquitously expressed during embryonic development in a stage- and tissue-specific manner, indicating its involvement in morphogenesis.[8] No GPC3 expression is detected in normal adult liver.[38] In contrast, upregulation of GPC3 has been reported in HCC in various studies, indicating its potential role as a biomarker in HCC.[6,39,40] Besides in HCC, GPC3 upregulation has been reported in other malignant tumors, including melanoma, ovarian cancer, and embryonal tumors such as neuroblastoma, and Wilm's tumor.[24,26] Microarray analysis of 4387 tissue samples revealed overexpression of GPC3 in 63.6% HCC (140/220), 54% squamous cell carcinoma of the lung (27/50), 52% testicular nonseminomatous germ cell tumors (32/62), and 52% liposarcoma (15/29).[25]

During embryonic development, the normal biological functions of GPC3 include negative regulation of cellular growth and modulation of cell differentiation and morphogenesis through binding with Wnt, Hedgehog signaling

FIGURE 2 Schematic diagram of GPC3 involvement in HCC development. GPC3 functions in HCC progression by stimulation of Wnt signaling, interaction with growth factors (FGF2, BMP-7, HGF, and TGF-β2), stimulation of macrophage recruitment, and promotion of EMT.

BMP-7, bone morphogenetic protein-7; BMPR, bone morphogenetic protein receptor; EMT, epithelial–mesenchymal transition; FGF2, fibroblast growth factor-2; FGFR, fibroblast growth factor receptor; GPC3, glypican-3; HCC, hepatocellular carcinoma; HGF, hepatocyte growth factor; HGFR, hepatocyte growth factor receptor; TAMs, tumor-associated macrophages; TGF-β2, transforming growth factor-β2; TGFR, transforming growth factor receptor.

proteins, and growth factors such as FGF through its HS side chains.[41–43] Loss-of-function mutation of *GPC3* has been shown to lead to Simpson–Golabi–Behmel syndrome (SGBS), an X-linked disorder that manifests as prenatal and postnatal overgrowth with visceral and skeletal anomalies.[44] SGBS-associated *GPC3* gene mutations include exonic deletions, frameshift, nonsense, splice, missense mutations, and exonic duplications.[45,46] Different studies have reported no correlation between the type or location of *GPC3* mutation and phenotype of SGBS patients, indicating that SGBS is caused by a nonfunctional GPC3 protein.[46,47] These loss-of-function mutations may lead to alterations in Wnt signaling, Hedgehog pathway, BMP signaling, FGF signaling, etc., and finally prenatal and postnatal overgrowth.[48–50] Moreover, GPC3-deficient mice present a phenotype that mimics the clinical manifestations of SGBS, including somatic overgrowth, which indicates that GPC3 is involved in developmental regulation.[51] And it has been demonstrated that the overgrowth of GPC3-deficient mice is attributable, at least in part, to the upregulation of Hedgehog signaling, indicating that GPC3 acts as a negative regulator of Hedgehog signaling during embryonic development.[42]

4 | MECHANISMS UNDERLYING GPC3 INVOLVEMENT IN HCC PROGRESSION

GPC3 is differentially expressed during the invasive growth of liver cancer, which suggests that GPC3 is potentially involved in HCC development.[52] Moreover, GPC3 knockdown results in the inhibition of HCC cell migration and invasion, which suggests the involvement of GPC3 in HCC metastasis and invasion.[53] In Figure 2, we summarize the

mechanisms of GPC3 involvement in HCC progression, including stimulation of Wnt signaling, interaction with growth factors, stimulation of macrophage recruitment, and promotion of epithelial–mesenchymal transition (EMT).

4.1 | Wnt signaling

Activation of the canonical Wnt signaling pathway is one of the most frequent molecular events associated with HCC progression. This signaling pathway is normally triggered by the binding of Wnt to two coreceptors: Frizzled, and low-density lipoprotein receptor-related protein 5/6 (LRP5/6).[54] Wnt binding induces the accumulation of the transcription factor β-catenin, which subsequently migrates to the nucleus and drives the expression of several genes, some of which are involved in promoting cell proliferation and survival. The Wnt/Frizzled/β-catenin pathway is activated in approximately 50% of HCCs, and up to 95% of HCCs exhibit potential Wnt/Frizzled-activating events.[55]

Previous work has clearly established that GPC3 modulates cell-surface signaling by acting as a coreceptor or storage site for Wnt proteins.[23] GPC3 can potentially activate Wnt signaling by increasing the amount of Wnt at the cell membrane, binding to both Wnt and its receptor Frizzled, stimulating the formation of signaling complexes, and through these actions, GPC3 can facilitate and stabilize the interaction of Wnt and its receptor Frizzled.[43] In canonical Wnt signaling, LRP5/6 forms a complex with Wnt and Frizzled, and thus LRP5/6 is likely to be a part of the GPC3-containing complex.[23] A complex including GPC3, Wnt, and Frizzled (LRP5/6) is then endocytosed, which results in the stimulation of the downstream signaling pathway. During the above signaling, not only the side chains, but also the core protein of GPC3 could interact with Wnt, thus facilitating the complex formation.[23] Besides interaction with Wnt, the HS side chains of GPC3 could interact directly with Frizzled and thereby facilitate the formation of signaling complexes.[55] Moreover, GPC3 expression was shown to be closely correlated with nuclear/cytoplasmic localization of β-catenin, consistent with the role of GPC3 in the activation of the Wnt signaling pathway.[56]

4.2 | Cell growth and growth factors

GPC3 has been demonstrated to interact with growth factors, act as a coreceptor and modulate growth factor activity through its HS chains, and, ultimately, stimulate cell growth.[5] Consistent with this, Akutsu et al. reported that small interfering RNAs (siRNAs) mediated GPC3 knockdown resulted in the downregulation of an array of growth factors at both the mRNA and protein levels in HCC cells.[56]

FGFs are a family of broad-spectrum growth factors that affect myriad cellular activities. The signaling pathways that are activated by FGF2 binding to the FGF receptor include the ras-raf-MAPK, PLCγ/PKC, and PI3K/AKT pathways.[57] In HCC cells, GPC3 binds to FGF2 through the HS side chains and might function as a coreceptor for FGF2.[41] Lai et al. reported that sulfatase 2 (SULF2), a heparin-degrading endosulfatase, increased GPC3 expression and promotes FGF signaling in HCC cells.[58]

Genetic studies have revealed that the hepatocyte growth factor (HGF)/c-Met pathway modulates HCC progression.[59,60] Gao et al. demonstrated that GPC3 functions in HCC cell migration and motility through HS chain-mediated cooperation with the HGF/c-Met pathway.[61] HCC cells treated with HS20, which targets the HS chains of GPC3, exhibited diminished capacity for HGF-induced migration and motility.[62] However, antibody targeting of the core protein of GPC3 has no effect on HCC motility.[62]

The transforming growth factor (TGF) superfamily, which includes TGF-β, activin, and BMP, modulates numerous cellular responses, including cell proliferation and apoptosis.[63] TGF-β signaling is mediated by the binding of TGF-β with TGF-β receptors, which in turn activates the downstream SMADs and associated complexes. Sun et al. discovered that GPC3 suppression in HCC cells leads to enhanced TGF-β2 expression and thereby inhibits cell proliferation, arrests cell cycle progression, and induces replicative senescence.[64] Midorikawa et al. demonstrated that GPC3 overexpression inhibits BMP7 signaling and modulates BMP7 activity through the SMAD pathway, and thus promotes cell proliferation.[5] However, GPC3 expression did not alter the effects of TGF-β1,[65] TGF-β3,[5] and BMP4 on HCC cell growth.[65]

4.3 | Macrophage recruitment

Macrophages are the most common immune cells in solid tumors and are generally classified as tumor-associated macrophages (TAMs), which play a pivotal role in the tumor microenvironment.[66] GPC3 membrane expression has been reported to stimulate the recruitment of macrophages that are likely to function as TAMs into tumor tissues.[67] Takai et al. examined liver tissues from 30 HCC patients and found that GPC3 membrane expression contributed to the recruitment of migrating macrophages into the HCC liver tissues while no correlation between GPC3 staining and the number of resident macrophages (i.e., Kupffer cells) was observed.[68] This finding was further confirmed by comparing the increase of macrophages between xenografts prepared using GPC3-transfected HCC cell lines and GPC3-nonexpressing cell lines.[68] Additionally, the results of genome-array analyses indicated that the macrophages that were recruited into GPC3-overexpressing HCC were M2-polarized TAMs, which function in angiogenesis and matrix remodeling.[67] Intratumoral M2-polarized TAMs promote tumor progression and metastasis by performing M2 macrophage functions, and therefore, are associated with poor prognosis.[69] Thus, GPC3 potentially contributes to HCC progression and metastasis through its influence on the function of TAMs.

4.4 | EMT

EMT is a crucial event in the tumor invasion process. In EMT, epithelial cell layers lose polarity and cell–cell contacts, and the cytoskeleton undergoes drastic remodeling.[70] EMT has been widely reported to be associated with the invasiveness and migratory ability of malignant tumors, including esophageal carcinoma, gastric carcinoma, HCC, colorectal cancer, and pancreatic cancer.[71] Among the various characteristics associated with EMT, E-cadherin inhibition is a significant hallmark. E-cadherin inhibition causes the primary malignant cells to migrate out of their primary site, to degrade surrounding extracellular matrix, and finally to migrate into the blood vessels and to lead to the invasion of secondary organs.[72] Qi et al. and Wu et al. have demonstrated a negative correlation between GPC3 and E-cadherin expression in HepG2 cells.[53,73] Additionally, Wu et al. reported that HCC tumor tissues with GPC3 overexpression showed low levels of E-cadherin.[73] Other EMT-related proteins (such as Snail and Slug) and migration-related proteins (matrix metalloproteinase 2 and 9) were decreased in GPC3-silenced HepG2 cells.[53] Taken together, these findings indicate that GPC3 overexpression promotes EMT in HCC cells and thereby enhances tumor progression and metastasis.

5 | REGULATION OF GPC3 EXPRESSION AND FUNCTION

As has been described, GPC3 is potentially involved in HCC development through various mechanisms. Therefore, it is of great clinical significance to understand the regulatory mechanism of GPC3 expression and function during HCC tumorigenesis. The various regulators of GPC3 expression are described in Figure 3.

5.1 | c-Myc

c-Myc is a transcription factor that plays a major role in >50% of all human tumors, regulating cell growth, proliferation, and differentiation. Li et al. demonstrated that GPC3 is a direct transcriptional target of c-Myc and that c-Myc overexpression increases GPC3 levels.[74] Mutational analyses have identified c-Myc-binding sites within the GPC3 promoter, indicating that c-Myc can directly activate GPC3 transcription.[74] Conversely, increased levels of GPC3 also elevated c-Myc expression, thus forming a positive-feedback signaling loop.

5.2 | Hippo/Yes-associated protein (YAP) pathway

YAP is a key downstream effector molecule in the classical Hippo pathway, which plays a critical role in regulating cell size, organ volume, tissue regeneration, and cancer development.[75] YAP is overexpressed in HCC and has been

FIGURE 3 Regulation of GPC3 expression and function. Reported regulators of GPC3 expression and function include (A) c-Myc and YAP, (B) ZHX2, (C, D) miRNAs, (E) SULF2, and (F) soluble GPC3. Solid lines indicate an inducible effect, while dashed lines indicate an inhibitory effect.
BMP-7, bone morphogenetic protein-7; FGF, fibroblast growth factors; GPC3, glypican-3; HGF, hepatocyte growth factor; SULF2, sulfatase 2; TGF-β2, transforming growth factor-β2; YAP, Yes-associated protein; ZHX2, zinc-fingers and homeoboxes 2.

identified as an oncogene that initiates and/or promotes HCC development.[76] Until now, several studies have indicated that GPC3 may be one of the target genes of YAP.[77–79] Li et al. observed that HCC tissues with higher YAP activation showed higher GPC3 expression, indicating a positive correlation between YAP activation and GPC3 expression.[78] Additionally, a recent study has revealed that $\alpha 2\beta 1$ integrin modulates the YAP signaling cascade in HCC and that knockdown of the *integrin-α2* gene led to significantly decreased expression of four YAP "target genes," including GPC3.[77] Pending further validation, these results indicate that GPC3 might be a downstream target of YAP in HCC.

5.3 | Zinc-Fingers and homeoboxes 2 (ZHX2)

ZHX2 belongs to the ZHX-family of transcription factors that are ubiquitously expressed and localized in the cell nucleus.[80] In normal adult liver, ZHX2 functions as a transcriptional repressor of the oncofetal genes *AFP*, *H19*, and *GPC3*.[81] In vitro analyses have demonstrated that the inhibitory effect of ZHX2 on *GPC3* transcription is through binding with its core promoter.[82] In HCC, *ZHX2* is often silenced by DNA methylation.[83] Furthermore, reduced nuclear ZHX2 expression might be responsible for GPC3 reactivation in HCC; accordingly, immunohistochemical staining of HCC tissues revealed an inverse correlation between nuclear ZHX2 and GPC3 expression.[82] However, the inhibitory effect of ZHX2 on GPC3 reactivation has only been demonstrated in vitro. In vivo studies need to be performed for further clarification.

5.4 | Sulfatase

The sulfation state of HS chains is critical for growth factor binding, and HS chains are potential substrates for desulfation at the 6-O position by human sulfatase.[58] In HCC, two kinds of sulfatases have been identified to be involved in tumorigenesis, sulfatase 1 (SULF1) and SULF2. SULF1 desulfates HS side chains and decreases heparin-binding growth factor signaling in HCC, thus acting as a tumor suppressor.[84] However, SULF2 functions as an oncogenic protein in HCC in part by upregulating FGF signaling in a GPC3-dependent manner.[58] Additionally, SULF2 was reported to increase cell-surface GPC3 and Wnt3a expression, increase GSK 3β phosphorylation, stabilize β-catenin, induce Tcf/Lef

transcriptional activity, and upregulate downstream Cyclin D1 expression in HCC both in vitro and in vivo.[85] During the above process, SULF2, GPC3, and Wnt3a associate in a possible ternary complex, and the activation of Wnt/β-catenin signaling by SULF2 in HCC is GPC3 dependent.

Although the precise mechanism is still unknown, Lai et al. hypothesized that there might be two different types of sulfated heparan sulfate glycosaminoglycans at the cell membrane or in the extracellular matrix.[86] One acts as a sulfation-dependent coreceptor of heparin-binding growth factors, and is a substrate for SULF1. Thus, enhanced SULF1 activity may lead to desulfation of HS side chains of the coreceptor heparan sulfate glycosaminoglycans and termination of heparin-binding growth factor signaling. The other one acts as a storage site for the sequestration of heparin-binding growth factors, and is a substrate for SULF2. Thus, enhanced activity of SULF2 may release heparin-binding FGF from the GPC3 storage sites and increase its availability for signaling.

We hypothesize that two mechanisms may account for GPC3 upregulation after SULF2 function. The first is that after function by SULF2, the "storage" class of GPC3 may be changed to the "coreceptor" class, and these molecules might participate in the activation of Wnt signaling, growth factor binding, etc. The second is that after SULF2 activity, enhanced levels of growth factors released from GPC3 may activate more GPC3 to facilitate their binding to corresponding receptors. However, the precise mechanism underlying the oncogenic effect of SULF2 in HCC needs to be elucidated.

5.5 | MicroRNAs (miRNAs)

miRNAs are small single-stranded, noncoding functional RNAs approximately 18–24 nucleic acids long. As negative regulators, miRNAs suppress mRNA expression at the posttranscriptional level by binding to the 3′-untranslated region (3′-UTR) of target mRNAs through base pairing, which results in target mRNA cleavage or translation inhibition. Maurel et al. screened a library of 876 miRNAs and identified five miRNAs that regulate GPC3 expression: miR-96 and miR-1271, which displayed negative regulation; and miR-129-1-3p, miR-1291, and miR-1303, which played inducing roles.[87,88] Further analysis revealed that miR-1271 expression is inversely correlated with GPC3 mRNA levels and that miR-1271 inhibits the growth of HCC cells in a GPC3-dependent manner.[87] Conversely, miR-1291 causes an increase in GPC3 mRNA expression by silencing inositol-requiring enzyme-1α, a negative regulator of GPC3 mRNA expression.[89] Additional studies have reported that miR-520c-3p and miR-219-5p exert tumor-suppressive effects in HCC by negatively regulating GPC3 expression: miR-520c-3p inhibited HCC growth by inducing HCC cell apoptosis, whereas miR-219-5p induced cell cycle arrest at the G1-to-S transition and markedly inhibited HCC cell proliferation.[90,91]

5.6 | Soluble GPC3 protein

Zittermann and colleagues transfected HCC cells with lentivirus to obtain soluble GPC3, which lacks the GPI anchor, and investigated its effect on cell growth and signaling.[92] The results showed that the soluble form of GPC3, which was secreted by infected HCC cells, might function as a dominant-negative protein and compete with endogenous GPC3 in the binding of downstream molecules. Thus, soluble GPC3 inhibited the in vivo growth of HCC cell lines by blocking protumorigenic signaling pathways, including blockade of the Wnt signaling pathway and the activity of heparin-binding growth factors (including FGF and HGF).[93] Feng et al. cultured HepG2 cells transfected with an expression plasmid for soluble GPC3 and detected a dose-dependent inhibition of cell proliferation by soluble GPC3, which confirmed the findings of Zittermann et al.[92,93]

6 | DIAGNOSTIC VALUE OF GPC3 IN HCC

As an oncofetal antigen, GPC3 is highly expressed in >70% of HCCs, but not in benign hepatic lesions, hepatic cirrhosis, hepatitis, or healthy adult tissues.[6] Not only in HCC tissue, but also serum GPC3 levels have been reported to be upregulated in HCC patients.[94]

6.1 | Tissue expression of GPC3

In 1997, Hsu et al. first described the abnormal expression of *MXR7* (later confirmed as *GPC3*) mRNA in 74.8% (143/191) of HCC tissues, as compared with expression only in 3.2% (5/154) of normal nontumor liver tissues.[39] Similar results were later obtained by Zhu et al., who reported that *GPC3* mRNA expression was upregulated in 83% of HCC tissues (25/30) as compared with the expression in focal nodular hyperplasia (FNH), liver cirrhosis, and normal liver tissues.[40] Since then, *GPC3* mRNA expression in HCC tissues has been investigated by a series of studies, and 55.7–100% of HCC tissues have been reported to express high levels of *GPC3* mRNA.[5,95] Similar results were obtained in HCC patients with hepatitis C virus (HCV) infection. A recent study enrolled 105 HCC patients with HCV infection and showed overexpression of *GPC3* mRNA in 86 patients (81.9%).[96] Additionally, in a study by Llovet et al., patients with HCV cirrhosis showed an 18-fold increase of *GPC3* mRNA in HCC tissues ($n = 20$) compared with that of dysplastic nodules ($n = 17$), indicating the discriminative value of GPC3.[11] These findings indicate that *GPC3* mRNA might serve as a tissue marker for the diagnosis of hepatitis B virus (HBV) /HCV-related HCC.

At the protein level, Capurro et al. detected GPC3 expression in 72% of HCC patients (21/29) through immunohistochemical staining, but detected no GPC3 expression in patients with a healthy liver or benign liver diseases (0/38).[6] In most HCC patients exhibiting GPC3 expression, GPC3 is mainly localized at the cell membrane and the cytoplasm.[4] In Table 1, we summarize the studies on GPC3 expression in surgical HCC tissues in which ≥100 HCC patients were enrolled; in these studies, GPC3 was reported to be expressed in 63–91% of the patients with HCC. In patients with HCV infection, immunohistochemistry results showed a 100% positivity of GPC3 staining in HCC ($n = 22$) compared with that in dysplastic nodules (0%; $n = 14$), indicating the diagnostic value of GPC3.[11] Different studies have reported that tissue GPC3 expression is significantly associated with HCV infection in HCC patients.[97,98] However, the tissue GPC3 expression pattern in nonviral HCC remains to be clarified.

GPC3 expression has been examined not only in surgical HCC samples, but also in fine-needle biopsies: Li et al. investigated needle biopsies from 179 patients with HBV-related HCC and detected GPC3 expression in 74.9% of the specimens.[10] However, it has to be noted that results from fine-needle biopsies may be misleading, and one has to be cautious while translating these results into clinical use.

The current literature indicates that GPC3 can serve as a highly specific diagnostic marker for HCC. However, the high heterogeneity presented by HCC has engendered the hypothesis that an optimal test for HCC will be based on the concurrent measurement of at least two highly specific tissue markers, and panels of three markers have been suggested for HCC diagnosis; the relevant studies on marker panels are listed in Table 2. The most frequently utilized HCC diagnostic marker panel combines GPC3 with heat shock protein 70 and glutamine synthetase (GS), and the sensitivity and specificity of HCC diagnosis for this panel are 58.7%–72% and 100%, respectively.[99–101] Other diagnostic marker panels combine GPC3 with arginase-1 (Arg-1) and hepatocyte paraffin-1 (HepPar-1) or GPC3 with CD34.[102,103] However, it has to be noted that three studies listed in Table 2 are based on needle biopsies, and specific caution should be paid when considering these results.

6.1.1 | Evaluation of GPC3 immunohistochemistry results

To accurately evaluate the tissue GPC3 protein expression in different studies, we recommend that the comprehensive scoring scale proposed by Takai et al. be adopted while reporting immunohistochemistry results.[104] The scoring system is composed of three elements, including positive cell rate, staining intensity, and staining pattern. The positive cell rate is graded on a scale of 0 to 3+: 0 (<5% tumor cells were stained positive), 1+ (5–10%), 2+ (10–50%), and 3+ (>50%). The staining intensity is classified as weak staining, moderate staining, and strong staining. The staining pattern is graded on a scale of I–III based on whether the cell membrane manifests as incomplete or complete circumferential staining (I: globally incomplete, II: generally incomplete with some complete staining, and III: generally complete with some incomplete staining). Additionally, the magnification should be indicated while reporting the results. This will enable comparative analyses of the immunohistochemistry results of GPC3 expression in different studies.

TABLE 1 Clinical Studies Related to Glypican-3 Expression in HCC Tissues from ≥100 Patients

Reference	GPC3 expression	Detection methods	Cut-off value	HCC tissues			Non-HCC liver	
				Etiology	No.	Positive rate (%)	No.	Positive rate
Man et al.[162]	mRNA	RT-PCR	-	-	100	90	21	19% positive in ICC
Saber et al.[99]	mRNA	RT-PCR	-	100% HCV (+)	105	81.9	-	-
Hsu et al.[42]	mRNA	Northern blot	-	-	191	74.8	154	3.2% positive in non-tumor liver
Cui et al.[127]	Protein	IHC	10% of tumor cells	100% HBV (+)	104	71.2	-	-
Shirakawa et al.[4]	Protein	IHC	-	27.1% HBV (+); 57.9% HCV (+)	107	81.3	-	-
Wang et al.[163]	Protein	IHC	-	-	111	75.7	110	0 in hepatocellular adenoma, FNH, and large RN
Gong et al.[164]	Protein	IHC	-	46.3% HBV (+) 4.4% HCV (+)	136	75.7#	103	18.4% in dysplastic nodules
Zhang et al.[165]	Protein	IHC	-	100% HBV (+)	147	87.1	25	0 in ICC
Fu et al.[125]	Protein	IHC	-	-	160	84*	-	-
Jeon et al.[126]	Protein	IHC	5% of tumor cells	78.4% HBV (+) 8.1% HCV (+)	185	91	-	-
Yorita et al.[101]	Protein	IHC	20% of tumor cells	30.9% HBV (+) 42.8% HCV (+)	194	77	-	-
Baumhoer et al.[28]	Protein	IHC	-	-	220	63.6	119	9.2% in nonneoplastic liver
Pan et al.[166]	Protein	IHC	-	83.7% HBV (+)	300	90	-	-
Feng et al.[131]	Protein	IHC	-	-	346	84.7	-	-
Liang et al.[167]	Protein	IHC	-	87.6% HBV (+) 1.7% HCV (+)	362	63.0	-	-
Li et al.[9]	Protein	IHC	-	-	426	80	-	-
Yan et al.[168]	Protein	IHC	-	76% HBV (+)	757	65	99	2% in ICC

#The amount of reactivity was graded as 1+ (<10% of cells) in 9.6% cases, 2+ (10–25% of cells) in 16.2% cases, and 3+ (>25% of cells) in 50% cases.
*The immunohistochemical staining was graded as low expression (≤25% positive staining tumor cells) in 16% HCC patients and high expression (≥26% positive staining tumor cells) in 68% HCC patients.

HCC, hepatocellular carcinoma; GPC3, glypican-3; RT-PCR, reverse transcription polymerase chain reaction; ICC, intrahepatic cholangiocarcinoma; HCV, hepatitis C virus; IHC, immunohistochemistry; HBV, hepatitis B virus; FNH, focal nodular hyperplasia; RN, regenerative nodules.

TABLE 2 Diagnostic tissue markers used for HCC diagnosis

Reference	No. of HCC	Diagnostic tissue markers	Diagnosis	Detection methods	Sensitivity (%)	Specificity (%)
Di et al.[102]	32[a]	HSP70+GPC3+GS (≥2 positive)	eHCC-G1	IHC	72	100
Di et al.[103]	92[b]	HSP70+GPC3+GS (≥2 positive)	HCC	IHC	58.7	100
Timek et al.[105]	29[c]	Arg-1+HepPar-1+GPC3 (≥2 positive)	HCC	IHC	79.3	–
Tremosini et al.[104]	40[d]	HSP70+GPC3+GS (≥2 positive)	HCC	IHC	60	100
Enan et al.[106]	60[a]	CD34 + GPC3	HCC	IHC	82	100

[a]Surgical tissue.
[b]Core needle biopsy using 18-19-gauge needles.
[c]Fine-needle aspiration.
[d]Biopsy using a 20-gauge or 18-gauge needles.
All the five studies defined immunoreactivity as "positive" if at least 5% of the lesional hepatocytes showed positive GPC3 staining.
HCC, hepatocellular carcinoma; HSP70, heat shock protein 70; GPC3, glypican-3; GS, glutamine synthetase; eHCC-G1, early and grade 1 hepatocellular carcinoma; IHC, immunohistochemistry;
Arg-1, arginase-1; HepPar-1, hepatocyte paraffin-1.

6.2 | Serum levels of GPC3

As has been mentioned, GPC3 can be released from the cell surface, and this occurs at the level of the GPI anchor. Thus, serum GPC3 level has been postulated to serve as a potential indicator of HCC. Serum GPC3 levels in HCC patients have been measured using enzyme-linked immunosorbent assay (ELISA), and the percentage of positive cases reported in HCC patients range from 36.1 to 95%.[105,106] Besides the serum GPC3 protein level, the mRNA level of GPC3 in peripheral blood has been measured, and the reported percentage of positive cases of circulating *GPC3* mRNA in HCC patients ranges from 28 to 100%.[106,107] In patients with HCV infection, HCV infection was reported to be associated with serum GPC3 N-terminal subunit antigen levels in HCC patients ($n = 115$).[108] Nault et al. enrolled 295 patients with alcoholic cirrhosis and observed that serum levels of GPC3 were significantly increased in patients with advanced HCC than in patients without HCC or those with early HCC.[109] However, the difference in serum GPC3 levels between patients without HCC or those with early HCC was not statistically significant.[109]

Results from studies that enrolled ≥100 HCC patients are shown in Table 3. The circulating levels of GPC3 reported thus far differ substantially, not only in HCC patients (median, 0–108.7 ng/mL), but also in healthy controls (0–5.9 ng/mL) and liver cirrhosis/chronic hepatitis (CH) patients (0–66.4 ng/mL). Several reasons may account for these discrepancies: different detection methods administered, different antibodies used, inconsistent cut-off value, different etiologies of HCC, etc. Thus, one should be cautious when translating these results. Although differences exist among studies, most studies have reported that serum GPC3 expression was significantly higher in HCC patients than in healthy controls and patients with liver cirrhosis or CH. Additionally, the results of several meta-analyses have suggested that serum GPC3 is upregulated in HCC patients, which indicates the potential of using serum GPC3 level in HCC diagnosis.[110–112] Thus, further studies using the same antibodies and detection methods in cohorts with similar etiologies are required to achieve clarity regarding the diagnostic suitability of serum GPC3.

To summarize, we suggest that clinicians resort to GPC3 tissue expression (mRNA or protein) testing if it is required, as this result is more specific and has better predictive value than conventional tests, as has been confirmed in different studies. As to the serum level of GPC3, additional studies need to be performed for a more objective conclusion.

6.3 | GPC3-Targeted imaging modalities used in HCC

In addition to being considered a therapeutic target, GPC3 has been regarded as a highly favorable target in the molecular imaging of HCC. GPC3-targeted imaging in HCC performed thus far includes positron emission tomography (PET), magnetic resonance imaging, and near-infrared imaging.

In the case of PET, GPC3-targeting antibodies conjugated to ^{89}Zr were used, and these antibodies showed extremely high ability to differentiate HCC tissue from normal liver tissue. Yang et al. constructed a PET probe, ^{89}Zr -desferrioxamine-1G12, and investigated its HCC-binding capacity; the results showed that ^{89}Zr -desferrioxamine-1G12 accumulated selectively in subcutaneous HCC and orthotopic xenografts.[113] The results of in vivo studies highlight the probe's potential for clinical translation—for use in early detection of HCC. Sham et al. constructed the probe ^{89}Zr-αGPC3 and confirmed that it can be used for imaging HCC and qualitatively determining GPC3 expression through small-animal PET.[114] Although the use of GPC3 antibodies has shown promise, the long circulation times of monoclonal antibody (mAb) has limited its clinical utility. To address this shortcoming, Sham et al. created the αGPC3-F(ab')2 fragment and conjugated it to ^{89}Zr; the resultant αGPC3-F(ab')2-^{89}Zr probe showed specific HCC tumor-binding with a 11-h half-life, as compared with approximately 115 h for traditionally used mAb controls.[115] This shorter half-life enabled clear tumor visualization in PET at 4 h after probe administration. The accelerated blood clearance and diminished background liver uptake of the probe yielded high signal-to-noise ratios at early time points, although future optimization is warranted in the process of clinical translation.

For magnetic resonance imaging, an anti-GPC3-ultrasuperparamagnetic iron oxide probe for early HCC detection was constructed to serve as a specific magnetic resonance targeting contrast agent.[116] In vitro results showed that the probe was specifically taken up by GPC3-expressing HepG2 cells, and that the signal intensity in T2-weighted images of HepG2 cells incubated with the probe decreased drastically after 2 or 4 h.

TABLE 3 Summary of clinical studies on serum glypican-3 level for HCC diagnosis (n ≥ 100)

Reference	GPC3 expression	Detection method	Antibody vendor	Cut-off value (ng/mL)	HCC Etiology	HCC No.	HCC Value (ng/mL) Median, range / Mean ± SD	Healthy controls No.	Healthy controls Value (ng/mL) Median, range / Mean ± SD	LC/CH No.	LC/CH Value (ng/mL) Median, range / Mean ± SD
Yan et al.[169]	mRNA#	FQ-RT-PCR	-	-	-	100	76% (+)	-	-	40	ND
Yao et al.[170]	mRNA#	Quantitative fluorescent PCR	-	-	72.4% HBV(+)	123	70.7%(+)	30	ND	70/70	1.4%(+)(LC) ND (CH)
Pisit et al.[171]	Protein	ELISA	Thermo Fisher, Denmark	20	70% HBV/HCV(+)	100	46.3 (0–7826.6)	40	ND*	50/50	0(0–43.6) (LC) ND (CH)*
Qiao et al.[172]	Protein	ELISA	USCN, USA	20.68	94.1% HBV/HCV	101	29.3 ± 17.3	30	5.9 ± 5.5*	40/18	12.1 ± 9.7 (LC)* 10.0 ± 9.6 (CH)*
Haruyama et al.[111]	Protein	ELISA	Self-made	-	22.6% HBV(+); 40% HCV(+)	115	0.4 ± 0.5	25	0.1 ± 0.04*	9	0.1 ± 0.1*
Lee et al.[173]	Protein	ELISA	Cusabio, China	73	62.5% HBV(+); 15% HCV(+); 10.8% alcohol	120	75.8 (21.7–482.5)	-	-	40	66.4(2.3–66.4)*
Yao et al.[170]	Protein	ELISA	Cusabio, USA	-	72.4% HBV(+)	123	0–4000*	30	ND	70/70	0–95.1 (LC)* 0–0 (CH)*
Chen et al.[174]	Protein	ELISA	Self-made	25.25	-	155	99.9 ± 267.2	136	4.1 ± 31.7*	124/180	19.4 ± 50.9 (LC)* 10.5 ± 46.0 (CH)*
Yu et al.[175]	Protein	Chemiluminescent immunoassay	Self-made	30	-	192	108.7 ± 230.0	48	4.0 ± 7.7*	44	6.87 ± 9.1*
Yasuda et al.[176]	Protein	ELISA	BioMosaics, Canada	-	100% HBV/HCV (+)	200	0.9 (0.5–1.3)	-	-	200	1.2 (0.8–1.8)*
Jia et al.[177]	Protein	ELISA	R&D, USA	0.002	80.6% HBV (+)	283	0 (0–14.0)	162	0 (0–4.3)*	445	0 (0–12.5)
Li et al.[9]	Protein	ELISA	Cusabio, China	3.5	100% HBV (+)	605	12.6 ± 2.9 (AFP < 400) 20.2 ± 5.4 (AFP ≥ 400)	25	1.9 ± 1.0*	-	-

Circulating GPC3 mRNA was extracted from the peripheral blood of enrolled HCC patients and controls.

* P < 0.05, compared with the HCC group

HCC, hepatocellular carcinoma; GPC3, glypican-3; LC, liver cirrhosis; CH, chronic hepatitis; FQ-RT-PCR, fluorescent quantitative reverse transcription polymerase chain reaction; ND, not detectable; HBV, hepatitis B virus; HCV, hepatitis C virus; ELISA, enzyme-linked immunosorbent assay; AFP, alpha-fetoprotein.

Park et al. developed a GPC3-specific multifunctional nanoparticle and demonstrated its dual-imaging suitability.[117] The GPC3-specific nanoparticles enhanced pretreatment magnetic resonance imaging and enabled refined intraoperative HCC visualization through near-infrared fluorescence. Thus, the nanoparticle could potentially be used as a carrier to deliver molecules in tumor-targeted therapies and thereby improve patient outcomes.

Zhu et al. used the phage-display method to identify GPC3-binding peptides and then labeled these peptides with a near-infrared dye, Cy5.5.[118] The Cy5.5-peptide exhibited high tumor accumulation in GPC3-expressing tumor xenografts in mice. In HCC patients, the Cy5.5-peptide effectively differentiates tumor tissues from healthy liver tissues, which indicates that the peptide holds considerable potential for clinical translation.[119]

7 | PROGNOSTIC VALUE OF GPC3 LEVELS

Since 2009, when Shirakawa first reported that GPC3-positive HCC patients showed poorer survival as compared with GPC3-negative patients, the relationship between GPC3 expression and HCC prognosis has been clarified in various studies.[4] Wang et al. observed that higher tissue GPC3 expression was correlated with poorer prognosis in patients with HBV-related HCC ($n = 69$).[120] In the case of HCC patients who had received surgical resection, positive tissue GPC3 staining was correlated with poor overall survival, disease-free survival, and postoperative metastasis/recurrence.[98,121,122] However, conflicting results exist with regard to the prognostic value of tissue GPC3 expression and overall survival and disease-free survival in HCC patients after hepatectomy.[97,123] Additionally, in HCV-positive HCC patients, circumferential membranous GPC3 expression indicates shorter disease-free survival after surgical resection ($n = 71$).[98] Besides GPC3 tissue-expression level, serum GPC3 level was previously investigated for its prognostic value, and the results showed that an elevated level of preoperative serum GPC3 N-terminal subunit antigen was associated with shorter overall survival and disease-free survival after hepatectomy ($n = 115$, cut-off value: 185.52 pg/mL).[108] In HCC patients who had received liver transplantation, tissue GPC3 expression was previously identified as a negative prognostic factor for recurrence-free survival, overall survival, and disease-free survival.[124,125] However, Wang et al. reported no prognostic value of preoperative circulating *GPC3* mRNA level in HCC patients who had received liver transplantation.[126] The distinct clinicopathological characteristics and disease etiologies of the enrolled patients in these studies might partially account for the reported differences.

Recently, GPC3 has been used in combination with other factors to investigate prognostic indications. Yu et al. have reported that HCC tissue expression of GPC3 plus osteopontin expression acts as a negative prognostic factor for patients with HBV-related small HCC after curative resection.[127] The findings of Feng et al. have indicated that the HCC tissue expression of both GPC3 and CK-19 is correlated with local recurrence in HCC patients after surgical resection.[128]

8 | GPC3-TARGETED THERAPIES IN HCC

Because GPC3 is overexpressed in HCC cells, various immunotherapies targeting GPC3 have been under investigation, including GPC3-targeted antibody treatment, peptide/DNA vaccine treatments, immunotoxin use, and genetic therapies. GPC3 targeted therapies that have been reported are shown in Figure 4.

8.1 | GPC3-Targeting antibodies

8.1.1 | GC33

GC33 is a mAb against the C-terminal 30 kDa fragment of human GPC3. GC33 treatment inhibited tumor growth in both ectopic and orthotopic GPC3-positive HCC xenograft models.[129] For clinical use, Nakano et al. constructed humanized GC33 and confirmed that its antitumor efficacy is mainly attributable to antibody-dependent cytotoxicity,

FIGURE 4 Glypican-3-targeted therapies in hepatocellular carcinoma. GPC3-targeted therapies in hepatocellular carcinoma include (A) GPC3-targeting antibody, (B) GPC3-derived peptide/DNA vaccine, (C) immunotoxin, (D) genetic therapy, and (E) other therapies.

CAR-T cell, chimeric antigen receptor T cell; CIK, cytokine-induced killer cells; CTL, cytotoxic T-lymphocyte; DC, dendritic cell; GPC3, glypican-3; MHC, major histocompatibility complex; miRNA, microRNA; PE, *Pseudomonas* exotoxin; shRNA, short hairpin RNA; siRNA, small interfering RNA.

alteration or disappearance of extracellular matrices, and a marked increase in macrophages.[130,131] Phase I, dose-escalation studies on GC33 for advanced HCC patients have been conducted in USA and Japan ($n = 20$ and 13, respectively); both studies revealed that GC33 was well tolerated and that no dose-limiting toxicity was detected up to the highest planned dose.[7,132] In the American study, patients were assigned to receive GC33 at dose levels of 2.5 mg/kg ($n = 4$), 5 mg/kg ($n = 3$), 10 mg/kg ($n = 4$), or 20 mg/kg ($n = 9$) every week. The median time to progression was considerably higher in the high-GPC3 expression group than in the low-GPC3 expression group (26.0 vs. 7.1 weeks).[7] In the Japanese study, patients were assigned to receive GC33 at dose levels of 5 mg/kg ($n = 4$), 10 mg/kg ($n = 3$), or 20 mg/kg ($n = 6$) every week. Seven of the 13 patients showed stable disease (neither sufficient shrinkage to be defined as "partial response" nor sufficient increase to be defined as "progressive disease") after GC33 administration.[132] A double-blind, phase II trial of GC33 was recently reported for 185 advanced HCC patients who failed prior systemic chemotherapy.[133] Results revealed that GC33 did not show clinical benefit in this population although it was potentially indicated that a larger dose of GC33, and choosing patients with high GPC3 expression or its mediator CD16 might improve outcome. However, further clinical studies are still needed to validate this hypothesis.

8.1.2 | Other mAbs

Apart from GC33, other mAbs have been constructed. Phung et al. used high-throughput flow cytometry and identified YP7, a mouse mAb that exhibits high-binding affinity for GPC3.[134] YP7 recognizes a GPC3 C-terminal epitope that overlaps with the binding site of humanized GC33; it showed considerable antitumor efficacy in tests on HCC xenograft tumors in mice.[134]

Feng et al. generated HN3, a human heavy-chain variable-domain antibody that targets—with high affinity—a conformational epitope in the native form of GPC3 core protein.[135] The antitumor efficacy of HN3 has been reported to be potentially attributable to cell-cycle arrest at the G1 phase through YAP signaling.[135] Compared to YP7, HN3 showed more favorable characteristics as a therapeutic antibody platform for designing molecular-targeted agents against HCC.[136]

Besides the mAbs targeting the core protein of GPC3, HS20, a human mAb that recognizes the GPC3 HS chains, has been generated.[61] Because the HS chains of GPC3 are critical for Wnt/β-catenin signaling and HGF binding/c-Met activation, HS20 could both disrupt GPC3-Wnt3a interaction and inhibit HGF-mediated cell migration, motility, and 3D-spheroid formation, and thus suppress cell proliferation.[62]

8.2 | GPC3-Derived peptide/DNA vaccines

In 2004 and 2006, Nakatsura et al. identified the human leukocyte antigen (HLA)-A24-restricted cytotoxic T-lymphocyte (CTL) epitope GPC3$_{298-306}$ peptide and the HLA-A2-restricted epitope GPC3$_{144-152}$ peptide in HCC patients.[137,138] Subsequently, these peptides were used as vaccines in preclinical studies with mouse models, and the optimal schedules for clinical use were established.[139,140] Furthermore, marked tumor lysis was observed in a HCC patient immediately after vaccination of the GPC3 peptide, which indicated the rapid antitumor effect of the peptide vaccine.[141]

In 2012, a nonrandomized Phase I trial was reported on the vaccines of the above two peptides for patients with advanced HCC.[142] High tolerability of the vaccines was observed, and GPC3-specific CTL responses were detected in 30/33 patients. In a recently published Phase II, case-control study of these two peptide vaccines, the 1-year recurrence rate of GPC3-positive patients who received surgery/radiofrequency ablation plus vaccines was significantly lower than that of GPC3-positive patients who received only surgery.[143] This result indicated that HCC patients who received initial resection or radiofrequency ablation might benefit from adjuvant therapy of GPC3 peptide vaccines.

In addition to the two aforementioned peptide vaccines, other GPC3-derived CTL epitopes have been identified, including murine GPC3-1$_{127-136}$ peptide.[144] Moreover, five GPC3-derived long peptides that can induce HLA class II-restricted Th1-cell response have been identified, and the Th1-cell response induced by these peptides was found to be strongly correlated with prolonged overall survival.[145] Furthermore, one of the five peptides, GPC3$_{137-161}$, could be effectively cross-presented when encapsulated in liposomes, and it elicited both Th1-cell response and CTL response.[145] Although clinical trials are still lacking, we believe that these peptides hold considerable promise for use in improving GPC3 peptide-based immunotherapy for HCC.

Nakatsura and co-workers conducted a study to effectively enhance the antitumor reactivity of GPC3-derived epitope peptides in vivo, and reported that liposome-coupled GPC3 peptides induced peptide-specific CTLs at a lower dose than did a conventional vaccine emulsified in incomplete Freund's adjuvant.[146] Additionally, programmed death-1/programmed death ligand-1 blockade has been reported to augment the antitumor effects of the GPC3-derived peptide vaccine by increasing the immune response of vaccine-induced CTLs.[147] Through this work, Nakatsura and co-workers have provided a foundation for the clinical development of combination therapy involving the use of GPC3 peptide vaccines with other medications.

Apart from these peptide vaccines, Li et al. constructed a GPC3 DNA vaccine and found that it elicited a CTL response against HCC cell lines, inhibited tumor growth, and prolonged survival time.[148]

8.3 | Immunotoxins

Recently, antibody fragments have been fused with toxin fragments to generate a chimeric structure termed immunotoxin. *Pseudomonas* exotoxin (PE) A is a common toxin fragment used in immunotoxins, and PE can induce the suspension of protein synthesis and ultimately cell death after separation of the chimeric molecules.[149] Thus, immunotoxins are believed to trigger tumor regression through two mechanisms: antibody-induced inactivation of cell signaling and toxin-induced inhibition of protein synthesis.

Gao et al. constructed immunotoxins HN3-PE38 and YP7-PE38, and in vitro and in vivo results showed that the antitumor efficacy of HN3-PE38 was higher than that of YP7-PE38 against HepG2 tumors in mice.[150] The antitumor cytotoxicity of HN3-PE38 involved the inhibition of two signaling pathways: Wnt3a-induced β-catenin signaling and YAP signaling pathways. However, off-target effects and the neutralizing antibodies induced by PE38 resulted in dose limitations and reduced antitumor efficacy.[150]

A second-generation PE fragment, mPE24, has shown reduced off-target effects and strong tumor inhibition. Wang et al. constructed HN3-mPE24 and HN3-HN3-mPE24 and investigated their safety and antitumor efficacy in HCC xenograft mice.[151] Higher GPC3-binding ability, lower IC_{50} values, and higher maximal tolerated doses were measured for HN3-mPE24 than for HN3-HN3-mPE24. Furthermore, intravenous administration of HN3-mPE24 induced marked tumor regression and extended survival with reduced side effects in the HCC xenograft mouse model.[151] Although further clinical investigation is required, the current data identify GPC3-targeting immunotoxins as promising candidates for HCC therapy.

8.4 | Genetic therapies

Genetic therapies targeting GPC3 have been under investigation for the past few years, including treatments involving the use of molecules such as miRNAs, short hairpin RNAs (shRNAs), and siRNAs. As noted in Section 5.5, miRNAs targeting GPC3 have exhibited antitumor efficacy in HCC xenograft models. RNA interference by using a GPC3 shRNAs inhibited the proliferative and invasive ability of HCC cells and HCC xenograft tumors,[152] induced cell-cycle arrest in the G1 phase and apoptosis,[153] and altered biological responses (including downregulation of β-catenin and vascular endothelial growth factor, and upregulation of Gli1).[154] Moreover, GPC3 knockdown by using siRNAs inhibited HCC cell proliferation through YAP downregulation,[155] induced apoptosis,[156] suppressed HCC cell growth through TGF-β2 upregulation,[64] and downregulated cell invasion and migration probably through EMT inactivation.[53] To overcome the barriers to effective siRNA delivery in clinical application, recently, Wang et al. constructed a nanovector (NP-siRNA-GPC3 Ab) which demonstrated antitumor efficacy in vitro without notable cytotoxicity and markedly suppressed orthotopic HCC xenograft tumor growth in mice.[157] Although these in vitro and in vivo results are promising, additional research is required before their clinical translation.

8.5 | Other therapies

Besides being used directly for their antitumor activity, mAbs can be conjugated to the photosensitizing phthalocyanin dye IR700 in order to target cancer cells through exposure to near-infrared light. Photoimmunotherapy performed using both IR700-YP7 and IR700-HN3 has shown a therapeutic effect in tumor-bearing mice.[136] Furthermore, photoimmunotherapy by using IR700-YP7 combined with nab-paclitaxel drastically increased nab-paclitaxel delivery and enhanced the therapeutic effect.[158]

Other molecular-targeted therapies include the use of GPC3-targeted chimeric antigen receptor T cells (CAR-T cell) and dendritic cell (DC) based cancer immunotherapy. Gao et al. obtained GPC3-targeted CAR-T cell and confirmed their antitumor efficacy both in vitro and in HCC xenografts.[159] Dargel et al. identified an HLA-A2-restricted peptide ($GPC3_{367}$) and used peptide multimers to clone GPC3-specific T cells; here, primary CD8+ T cells expressing the transgenic T-cell receptor that specifically bound to GPC3 were identified, and these T cells killed GPC3-expressing hepatoma cells in vitro and slowed the growth of HCC xenograft tumors in mice.[160] Wang et al. constructed *GPC3*-transduced DCs and cocultured them with autologous cytokine-induced killer cells (CIKs).[161] These DC-GPC3-CIKs exerted a strong antitumor effect against GPC3-expressing HCC cells both in vitro and in vivo. The results of these studies have provided new insights that can aid the design of personalized adoptive immunotherapy for GPC3-positive HCC.

9 | CONCLUSION

Owing to the research conducted over the past 20 years, GPC3 has developed from a promising biomarker for detection of early HCC to an effective epitope for HCC-targeted therapies. Although the mechanisms underlying GPC3 involvement in HCC progression need to be clarified, it is certain that GPC3 functions in the general molecular pathways in HCC development. The overexpression of GPC3 in HCC cells makes it a biomarker of HCC with high diagnostic efficacy. When combined with AFP, the sensitivity and specificity can be elevated significantly. Excitingly, with the development of molecular imaging, the membrane expression of GPC3 of HCC cells has endowed it with great potential for clinical translation in HCC-targeting imaging. With the high tumor-to-liver ratio, we believe GPC3-targeted imaging will have a promising future in the pretreatment location/staging/planning of HCC, identification of surgical margin during operation, and monitoring of therapies. The promising in vitro and in vivo therapeutic results obtained thus far support an optimistic view with regard to the prospects of GPC3-targeting therapies markedly improving the clinical outcome in HCC. We believe that future research must focus on multitargeting strategies for HCC therapy. The findings of previous knockdown studies indicate that GPC3 gene deletion is not lethal to HCC cells, and anti-GPC3 antibodies are not adequately potent for curative treatment of HCC. Thus, to overcome this problem, combination therapies including chemotherapy (e.g., sorafenib treatment), antibody-drug conjugate (liposome) use, and CAR-T-cell adoptive therapy have been investigated. Another hurdle is the stability of GPC3 expression after targeted treatment: cells that survive treatment might lose GPC3 expression and thus gain drug resistance. To address this problem, additional future studies must be conducted on the regulation of GPC3 expression to obtain drugs that exhibit stronger tumor-suppression activity than do currently available GPC3-targeting drugs.

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