REVIEW

Critical analysis of the potential of targeting GPC3 in hepatocellular carcinoma

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Abstract: Hepatocellular carcinoma (HCC) is a leading cause of cancer-related deaths worldwide. The treatment options for patients with advanced HCC are limited, and novel treatment strategies are required urgently. Glypican-3 (GPC3), a member of the glypican family of heparan sulfate proteoglycans, is overexpressed in 72%–81% of HCC cases, and is correlated with a poor prognosis. GPC3 regulates both stimulatory and inhibitory signals, and plays a key role in regulating cancer cell growth. GPC3 is released into the serum, and so might be a useful diagnostic marker for HCC. GPC3 is also used as an immunotherapeutic target in HCC. A Phase I study of a humanized anti-GPC3 monoclonal antibody, GC33, revealed a good safety profile and potential antitumor activity, and a Phase II trial is currently ongoing. In addition, the authors' investigator-initiated Phase I study of a GPC3-derived peptide vaccine showed good safety and tolerability, and demonstrated that the GPC3 peptide-specific cytotoxic T-lymphocyte frequency in peripheral blood correlated with overall survival in HCC patients. A sponsor-initiated Phase I clinical trial of a three-peptide cocktail vaccine, which includes a GPC3-derived peptide, is also underway. GPC3 is currently recognized as a promising therapeutic target and diagnostic marker for HCC. This review introduces the recent progress in GPC3 research, from biology to clinical impact.

Keywords: GPC3, hepatocellular carcinoma, immunotherapy

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths worldwide.[1] HCC patients are often diagnosed at an advanced stage, and so the prognosis is often poor. Currently, surgery or locally ablative treatments such as percutaneous ethanol injection or radiofrequency ablation are the standard treatments for early-stage HCC. However, these treatments are no longer available and options are limited for most patients with advanced HCC.[2] Generally, transarterial chemoembolization or systemic chemotherapy is used. However, these therapeutic approaches are not curative in most patients. Sorafenib, a multi-targeted tyrosine kinase inhibitor, is the only drug that has significantly prolonged the survival of patients with advanced HCC;[3,4] therefore, it has become the standard agent for first-line systemic treatment. However, the incidence of adverse effects is high, and there are no effective second-line treatments for patients who do not respond to sorafenib. Therefore, new treatment strategies for patients with advanced HCC should be established.

To date, several immunotherapeutic clinical trials in patients with advanced HCC have been performed. These studies have shown feasibility and safety, but no dramatic clinical responses.[5,6] Nevertheless, some randomized controlled trials have shown the

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potential to reduce the risk of cancer recurrence in adjuvant settings.[6] Therefore, an immunotherapeutic approach is potentially an attractive treatment option for HCC.

Various tumor antigens for HCC have been identified and investigated as immunotherapeutic targets.[7] GPC3 is a member of the glypican family of heparan sulfate proteoglycans that are attached to the cell surface via glycosylphosphatidylinositol (GPI) anchors.[8] Mutations in *GPC3* cause Simpson–Golabi–Behmel syndrome,[9] which is an X-linked disorder characterized by pre- and postnatal overgrowth with visceral and skeletal anomalies. *GPC3*-deficient mice exhibited similar characteristics as Simpson–Golabi–Behmel syndrome patients.[10] GPC3 is overexpressed in 72%–81% of patients with HCC.[11–15] Therefore, GPC3 has been recognized as a potential immunotherapeutic target or diagnostic marker for HCC. This paper reviews the biology of GPC3 and discusses recent advances in GPC3-targeted HCC immunotherapy.

Tumor-associated antigens (TAAs) in HCC

TAA-specific immunotherapy is an attractive strategy because it is associated with fewer adverse events. Therefore, identifying appropriate TAAs is important for the development of TAA-specific cancer immunotherapies. Boon et al initially reported that MAGE-A was a human TAA in a melanoma patient, and that the human immune system could recognize TAA expressing-cancer cells as foreign bodies and exclude them.[16] Subsequently, a novel approach termed serological analysis of recombinant complementary DNA expression libraries (SEREX) was developed to identify TAAs.[17,18] Complementary DNA microarray technology is also useful for identifying novel cancer-associated genes and for classifying human cancers at the molecular level.[19,20] In HCC, some TAAs, such as AFP, MAGE-A, NY-ESO-1, SSX2, and telomerase reverse transcriptase, have been identified.[7] Although GPC3 is overexpressed in HCC,[11–15] it is not expressed in most normal adult tissues. Furthermore, GPC3-expression was correlated with poor prognosis in patients with HCC: GPC3-positive HCC patients had a significantly lower 5-year survival rate than GPC3-negative individuals (54.5% versus 87.7%; *P*=0.031).[15] These results suggest that GPC3 might be a promising target for cancer immunotherapy.

Biological aspects of GPC3
General considerations

Glypicans are a family of heparan sulfate proteoglycans. To date, six glypicans have been identified (GPC1 to GPC6)

in mammals, and two orthologs of the mammalian genes were identified in *Drosophila melanogaster* (Dally- and Dally-like).[8,21] Glypicans of all species are classified into two subfamilies according to their sequence homology.[21] In general, the function of glypicans is to regulate morphogenesis during embryonic development,[22] and mutations cause the overgrowth genetic disease Simpson–Golabi–Behmel syndrome.[23] Several recent studies have revealed that GPC3 is overexpressed in many cancers.

Structure and function of GPC3

GPC3 is a 580-amino acid protein (~60 kDa) that is encoded by nine exons on chromosome X (Xq26). Alternative splicing results in four variants that were isolated from the HepG2 cell line. Fourteen cysteine residues located in the core region are well conserved among glypicans, and contribute to the formation of a unique ternary structure via disulfide bonds. The amino-terminus contains a signal peptide sequence (residues 1–24), which is required for targeting to the cell surface. The carboxyl-terminus contains a hydrophobic region that is associated with the lipid bilayer of the Golgi apparatus. During the transport of GPC3 to the cell surface, the hydrophobic region is truncated by transamidase, and then covalently attached to a GPI anchor via the C-terminus of serine 560.[24] Therefore, the attachment of a GPI anchor is a key post-translational modification that regulates the cellular localization of GPC3.

GPC3 regulates both stimulatory and inhibitory signals through the binding of heparan sulfate chains to signaling molecules such as Wnt, Hedgehog, fibroblast growth factors, bone morphogenetic proteins.[25–31] The core protein also plays an important role for regulating the activity in Wnt and Hedgehog signaling.[27,28,32] Structural information regarding GPC3 is needed to understand these signaling mechanisms, but the three-dimensional structure of GPC3 is yet to be elucidated. Nevertheless, the crystal structure of *Drosophila* Dlp, an ortholog of the mammalian gene, is available.[33] Structural analysis of the Dlp core region revealed an elongated conformation with α-helix packing: this is a unique structure when compared with other proteins. Further structural studies of glypicans are necessary to understand their complex and multifunctional signaling pathways and their regulation of cancer cell growth.

GPC3 biology and disease

GPC3 is expressed in many embryonic tissues in addition to fetal liver and placenta.[34] The overexpression of GPC3 is observed in liver cancer, ovarian cancer, lung cancer, malig-

nant melanoma, and embryonal cancers such as neuroblastoma medulloblastoma and Wilms' tumor.[35–41] Capurro et al demonstrated that the binding of GPC3 to Wnt and Hedgehog activates signaling pathways that promote the growth of HCC cells.[27,28] Moreover, the knockdown of GPC3 using small interfering RNA and subsequent gene expression analysis revealed that suppressing GPC3 inhibited the transforming growth factor-β (TGF-β) receptor pathway and the subsequent growth of HCC cell lines.[42] These suggest that GPC3 is an important target for cancer therapy.[43,44]

It is noteworthy that GPC is a novel serological cancer marker.[12,45,46] Secreted circulating GPC3 is detected in the blood of cancer patients with HCC[11,45] and melanoma,[37,47]and the presence of soluble GPC3 correlates with cancer progression. However, because GPC3 is initially membrane-bound via a GPI anchor, it is currently unknown how GPC3 is secreted into the circulation. It was reported that GPC3 can be cleaved by Notum (α/β-hydrolase enzyme) and furin-like convertase,[48,49] releasing the N-terminal domain and full-length GPC3 from the cell surface.[50,51] Secreted GPC3 might be useful for cancer diagnosis.

GPC3 as a diagnostic marker for HCC
GPC3 expression in HCC at the messenger RNA or protein level

Several studies have suggested that GPC3 is a potential therapeutic target in liver cancer because it is overexpressed in HCC, but is not expressed or is expressed at only low levels in normal adult tissue.[52–54] Hsu et al performed pioneering work to identify GPC3 as a potential biomarker for HCC.[55] When GPC3 was compared with AFP, another established HCC marker, data revealed higher *GPC3* messenger RNA expression compared with serum α-fetoprotein (AFP), levels (71.7% versus 51.3%) based on the analysis of 113 patients with unicentric primary HCC. The authors also reported previously that *GPC3* is specifically overexpressed in HCC by analyzing complementary DNA microarrays containing 23,040 genes. The expression profiles of 20 HCC samples, corresponding noncancerous liver tissues, and various normal human tissues revealed that GPC3 was overexpressed specifically in HCC.[11]

Capurro et al confirmed increased GPC3 expression in HCC patients using a mouse monoclonal antibody (1G12) against a GPC3 C-terminal peptide.[12] Immunohistochemistry revealed that GPC3 was overexpressed in 72% of HCC samples. Therefore, GPC3 might also be useful as an ancillary tool during histopathological diagnostic processes to

distinguish HCC from cirrhosis, dysplastic nodules, and focal nodular hyperplasia-like nodules.[56]

GPC3 as a serum marker for HCC

Several studies have been performed to validate the diagnostic potential of GPC3 as a serum marker by developing methodologies such as enzyme-linked immunosorbent assays and radioimmunoassays.[45,57] Several antibody-based immunoassays have been developed to assess potential serum biomarkers. Using multiple serum markers, including AFP and protein induced by vitamin K absence or antagonists-II (PIVKA-II), might increase diagnostic accuracy. Although GPC3 is a cell-surface marker, it can be released into the serum by the lipase Notum, which cleaves the GPI anchor.[49] Specifically, Hippo et al reported that GPC3 is cleaved between Arg358 and Ser359, and that the N-terminal fragment of GPC3 is also released into circulation. They reported the usefulness of the N-terminal fragment of GPC3 for diagnosing early-stage HCC.[51] Therefore, GPC3 also exhibits diagnostic value as a serum marker.[57,58] Qiao et al compared the serum levels of three markers (GPC3, human cervical cancer oncogene [HCCR], and AFP) for diagnosing HCC in 189 patients (101 HCC, 40 cirrhosis, and 18 hepatitis cases and 30 healthy control donors). They reported that GPC3 was the most accurate diagnostic marker: using a cutoff of 26.8 ng/mL for the diagnosis of HCC, GPC3 had a sensitivity of 51.5% and a specificity of 92.8%. In addition, the simultaneous detection of three markers increased the sensitivity significantly to 80.2% higher than AFP alone.[58] In a meta-analysis comparing AFP and GPC3 as serum markers for HCC, the pooled sensitivities for AFP and GPC3 were 51.9% and 59.2%, and the pooled specificities were 94% and 84.8%, respectively.[59] This suggests that GPC3 and AFP are comparable serum markers. Serum GPC3 might be a useful tumor marker in patients with HCC. However, the biochemistry of serum GPC3 is yet to be elucidated, and so further studies are needed.

GPC3 as an immunotherapeutic target in HCC
Identification of human leukocyte antigen (HLA)-A2- or A24-restricted GPC3-derived epitope peptides

Identifying TAA-derived epitope peptides is the first step in the development of peptide vaccines. *HLA-A24* is the most common HLA class I allele in the Japanese population (60%).[60,61] Structural motifs of peptides bound to human HLA-A24 and BALB/c mouse H-2K^d are similar,[62,63] and the amino acid sequences of human and mouse GPC3 have 95% homology. These studies identified the mouse GPC3-derived

and K^d- restricted cytotoxic T-lymphocyte (CTL) epitope peptide GPC3$_{298-306}$ (EYILSLEEL) in BALB/c mice. This peptide-specific CTL showed specific cytotoxicity against GPC3-expressing or peptide-pulsed cancer cell lines, suggesting that GPC3 was highly immunogenic and could elicit effective antitumor immunity in mice. Importantly, there was no evidence of autoimmune reactions in the treated mice.[64] Because of the similarities in the peptide binding motifs between H-2K^d and HLA-A24, this peptide was applicable for immunotherapy in HLA-A24-positive patients.

HLA-A2 is also expressed in 40% of Japanese individuals, as well as other ethnic populations.[60,65] An HLA-A2-restricted GPC3$_{144-152}$ (FVGEFFTDV) peptide was also identified using *HLA-A2.1* transgenic mice.[58] A binding assay was performed, and it was reported that the HLA-A*02:01-restricted GPC3$_{144-152}$ (FVGEFFTDV) peptide could bind to HLA-A*02:06 and HLA-A*02:07. This suggests that HLA-A2-restricted GPC3$_{144-152}$ (FVGEFFTDV) might be effective in HLA-A*02:06 and HLA-A*02:07 patients.

These GPC3-derived peptide-specific CTLs could be induced from the peripheral blood mononuclear cells of HCC patients by in vitro stimulation with peptide. The adoptive transfer of these GPC3-derived peptide-specific CTLs reduced the mass of human HCC tumors implanted into non-obese diabetic/severe combined immunodeficiency mice.[66]

GPC3-targeted vaccine therapy

The authors recently completed an investigator-initiated Phase I clinical trial of GPC3-derived peptide vaccines to evaluate their safety, tolerability, and efficacy in patients with advanced HCC.[67] Thirty-three advanced HCC patients were enrolled and received escalating doses of GPC3-derived peptide vaccine (0.3, 1.0, 3.0, 10, and 30 mg/patient). On days 1, 15, and 29, peptides were administered in liquid form, emulsified with incomplete Freund's adjuvant by intradermal injection. GPC3$_{298-306}$ (EYILSLEEL) peptide was used in 17 HLA-A24-positive patients, and GPC3$_{144-152}$ (FVGEFFTDV) peptide was used in 16 HLA-A2-positive patients.

Dose-limiting toxicity and dose-specific adverse events were not seen, and GPC3-derived peptide vaccine treatment was well tolerated. One of the thirty-three patients was judged to have a partial response, whereas 19 patients exhibited stable disease after 2 months according to Response Evaluation Criteria In Solid Tumors (RECIST).[68] The disease control rate (partial response plus stable disease) was 60.6% after 2 months. The median time to tumor progression was 3.4 months (95% confidence interval [CI] 2.1–4.6), and the median overall survival was 9.0 months (95% CI 8.0–10.0).

Immunologically, the frequency of GPC3-peptide-specific CTL in the peripheral blood correlated with the overall survival of HCC patients. In the multivariate analysis, GPC3 peptide-specific CTL frequency was a predictive factor for overall survival. The median overall survival of all 33 patients was 12.2 months (95% CI 6.5−18.0) in patients with a high frequency of GPC3-specific CTLs compared with 8.5 months (95% CI 3.7−13.1) in individuals with a low frequency (*P*=0.033). Moreover, the infiltration of cluster of differentiation (CD)8-positive T-cells into HCC cells was confirmed.

Based on this Phase I study, a Phase II study of the GPC3-derived peptide vaccine is ongoing in an adjuvant setting (UMIN-CTR: 000002614). Forty-four patients with HCC who had undergone surgery or radiofrequency ablation were enrolled. The primary end points of this study were the 1- and 2-year recurrence rates, and the secondary end point was the immunological response. Patient enrollment has been completed, and the study is ongoing. An additional sponsor-initiated Phase I clinical trial of a three-peptide cocktail vaccine, which includes a GPC3-derived peptide, is also underway.

Anti-GPC3 antibody therapy

GPC3 has been suggested as a potential target for antibody-based therapy in liver cancer because of its high-level expression in HCC. The murine monoclonal antibody GC33, which binds specifically to the C-terminal region of GPC3 with a high affinity, caused significant antibody-dependent cellular cytotoxicity against HCC cells, and exhibited potent antitumor activity in xenograft models.[69–72] For the clinical application of GC33, a humanized GC33 was generated using complementarity-determining region grafting with the aid of both the hybrid variable region and two-step design methods. To improve the stability of the humanized GC33, it was further optimized by replacing the amino acid residues that might affect the structure of the variable region of its heavy chain.[73]

Because of these preclinical data highlighting the relevance of GPC3 as a potential therapeutic target in HCC, a first-in-man Phase I clinical trial to assess the safety, tolerability, and pharmacokinetics of GC33 in patients with advanced HCC was performed.[74] A total of 20 patients were enrolled, and were assigned to receive GC33 at one of four sequentially increasing dose levels (2.5, 5, 10, and 20 mg/kg) weekly by intravenous infusion. The tumor expression of GPC3 was examined in biopsied specimens using immunohistochemical staining. A total of 56% of the patients had a high total GPC3-staining score. This study provided the

initial clinical data regarding the safety profile and pharmacokinetic features of GC33, and revealed potential antitumor activity that might be associated with the expression of GPC3 in tumors. Stable disease was seen in four patients, all of whom exhibited high GPC3 expression. The median time to progression was significantly longer in patients with tumors expressing high levels of GPC3 than in patients with low GPC3 expression.

GC33 is now being assessed in Phase II clinical trials in second-line HCC patients who have progressed after one line of systemic therapy and whose tumors exhibit positive GPC3 immunohistochemical staining (NCT01507168). Additional antibodies that target GPC3 for HCC treatment, human (MDX-1414 and HN3) and humanized mouse (YP7) antibodies, are at different stages of preclinical development.[75] These trials will define the potential of GPC3 as a novel antibody therapy.

Potential of GPC3 for other cancers

GPC3 is also overexpressed in other malignant tumors, such as melanoma, Wilms' tumor, hepatoblastoma, yolk sac tumor, ovarian clear-cell carcinoma (CCC), and lung squamous cell carcinoma.[37-41] However, Kim et al reported that GPC3 is downregulated in lung cancer. Thus, the overexpression of GPC3 in lung cancer is controversial.[76] GPC3 has been investigated in some of these tumors as a potential immunotherapeutic target or diagnostic marker.

Melanoma

GPC3 messenger RNA and protein was identified in >80% of melanoma and melanocytic nevus patients.[39] In the authors' previous study, GPC3 protein was detected in the sera of 39.6% melanoma patients, but not in healthy donors. The positive detection of serum GPC3 was significantly higher than that of 5-S-cysteinyldopa and melanoma-inhibitory activity, both of which are well-known tumor markers for melanoma. Surprisingly, GPC3 could be detected even in patients with stage 0 in situ melanoma.[37] The combination of secreted protein acidic and rich in cysteine (SPARC) and GPC3 was also a useful tumor marker for melanoma: 66.2% of melanoma patients at stages 0−II exhibited positive SPARC or GPC3 expression.[47] This suggests that GPC3 is a novel tumor marker that is useful for the diagnosis of melanoma, particularly during the early stages.

Ovarian carcinoma

Ovarian CCC is the second most common epithelial ovarian carcinoma subtype in Japan. Ovarian CCC is associated with a poor prognosis and increased chemoresistance compared with other epithelial ovarian carcinoma subtypes.[77,78] GPC3 was expressed in ~40% of CCC patients, and there was a tendency toward poor progression-free survival in GPC3-positive patients at stage I.[79] GPC3 expression was responsible for CTL recognition, and subtoxic dose chemotherapy made tumor cells more susceptible to the cytotoxic effects of CTL.[80] A Phase II trial of a GPC3-derived peptide vaccine in ovarian CCC patients is ongoing (UMIN-CTR: 000003696), and some chemotherapy-refractory ovarian CCC patients have achieved a significant clinical response.[81]

Pediatric tumors

A Phase I trial using a GPC3-derived peptide vaccine for pediatric patients with hepatoblastoma, nephroblastoma, or yolk sac tumors is ongoing (UMIN-CTR: 000006357). The safety and optimal dose of GPC3 peptide vaccines for pediatric cancer patients has not yet been reported.

Conclusion

Although immunotherapy is a potentially attractive treatment modality, its antitumor effects in advanced HCC are not dramatic. GPC3 is overexpressed in HCC but its expression in most adult normal tissues is low. GPC3 expression is correlated with poor prognosis in HCC, suggesting it to be an ideal tumor antigen. GPC3 is thought to play a role in regulating cancer cell growth, although our structural and biological knowledge of GPC3 remain limited. Recent studies have shown the utility of GPC3 as a serum and immunohistochemical marker for the diagnosis of HCC. In addition, although studies assessing GPC3-targeted immunotherapies against HCC (such as vaccine and antibody therapies) have shown good safety and tolerability, sufficient clinical effects have not yet been observed. Further analysis and knowledge of GPC3 biology and its potential as an immunotherapeutic target are needed to allow the development of more effective GPC3-targeted cancer therapies. Although current GPC3-targeted immunotherapies for HCC are in the preclinical and clinical trial phases of development, they are expected to yield clinical success in the near future.

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