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Review Article

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Glypican-3 (GPC-3) for early diagnosis and target therapy

***Corresponding Author: Yan Li**

School of Pharmacy, Naval Medical University, 325 Guohe Road, Yangpu District, Shanghai, 200433, China.

Email: 912395665@qq.com

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Abstract

Liver cancer is one of the highest causes of cancer-related deaths worldwide, and China accounts for more than half of the new cases and deaths. It's highly malignant and progresses rapidly. Once diagnosed, it is often in the late stage of the disease with limited treatment options and bad prognosis. How to diagnose it in the early stage becomes a key factor in preventing disease progression. What's more, there are also limited treatment options. Liver resection or transplantation is a potential curative treatment, but only a small proportion of the patients are suitable for it. Specific biomarkers and molecular targets bring a new dimension to cancer personalized diagnosis and more-targeted treatment. Glypican-3 (GPC-3) as an oncofetal protein that attaches to the exocytoplasmic surface of the plasma membrane is mainly expressed in the specific tumor tissues. In recent years, great progress has been made for GPC3 in the diagnosis and treatment of liver cancer. The following review article will focus on GPC-3 as a new specific biomarker and molecular target for early diagnosis and target therapy in liver cancer.

Introduction

Cancer is a major public health problem in the world, and the incidence of liver cancer continues to rise every year [1]. As the incidence rates increases, liver cancer is one of the highest causes of cancer-related deaths worldwide [2], and China accounts for more than half of the new cases and deaths [3]. The most common type of primary liver cancers are hepatocellular carcinoma (HCC), which accounts for 80% of the cases [4], and cholangiocarcinoma, which accounts for 6% of the cases [5]. Hepatocellular Carcinoma (HCC) is the third most deadly type of cancer worldwide [6,7], and the fifth most prevalent malignancy [8]. This cancer occurs in the context of patients with underlying liver disease, such as liver fibrosis and cirrhosis associated with chronic viral infections [9]. In addition, metabolic disorders which are defined as Nonalcoholic Fatty Liver Disease/Nonalcoholic Steatohepatitis (NAFLD) and the metabolic syndrome, contribute even more to HCC than any other risk-factor including HCV infection, because of the high prevalence of metabolic disorders in the population [10,11]. Liver cancer has a very low overall survival rate [9,12], and the age-standardized 5-year relative survival rate was only 10.1% reported by the National Central Cancer Registry (NCCR) of China (diagnosed in 2003–2005 and followed until the end of 2010) [13]. Low survival rates are attributed to difficulties in early diagnosis and limited treatment options [14]. Most patients are usually asymptomatic until it's too late and diagnosed with advanced disease with abnormal symptoms often caused by metastasis, consequently missing the best time for therapy. In addition, due to the low selectivity and toxicity of chemotherapy drugs, it is highly malignant and progresses rapidly, and it is difficult to treat advanced liver cancer, and the treatment effect is poor with a bad prognosis. Liver resection or transplantation is a potential curative treatment, but only a small part of the patients are suitable for the operation [12], and necessary assessment of the tumor invasion level before operation is crucial with imaging techniques. Specific biomarkers and molecular targets bring a new dimension to cancer personalized diagnosis and more-targeted treatment. Glypican-3 (GPC-3) a heparan sulfate proteoglycan of the glypican family attaches to the cell surface via the glycosyl-phosphatidylinositol anchor, and it is a specific biomarker for HCC detection. Many studies have shown that GPC-3 is expressed in 64% to 90% of HCC, but not in normal liver or benign tumors such as hepatic adenomas [15,16]. The following review focuses on GPC-3 as an ideal biomarker for precision diagnosis and target therapy for liver cancer.

Glypican-3 (GPC-3)

Glypican-3 (GPC3), a member of the glypican family is an oncofetal protein which attaches to the exocytoplasmic surface of the plasma membrane via a glycosyl-phosphatidylinositol an-

chor [17,18]. GPC3 expresses in a wide variety of tissues during embryonic development [19]. Moreover, it mainly expressed in HCC and melanoma, ovarian clear cell, carcinoma of Yolk Sac Tumor (YST), neuroblastoma, hepatoblastoma, Wilms tumor cells [20], but absent in normal adult tissues, cirrhotic liver, and benign lesions [21,22].

GPC3 has different forms with different locations, a non-cleaved form 70-kDa precursor core protein was found to be localized in the cytoplasm while a 40-kDa amino N-terminal cleaved protein was fond to be localized in the cytoplasm and at the extracellular side of hepatocyte membranes. Additionally, result of immunofluorescence found that the non-cleaved form of GPC3 co-localizes with Furin-Convertase in the Golgi apparatus [23]. What's more, the N-terminal protein can be cleaved from GPI anchoring site from the outer surface of the cell membrane and enter the bloodstream leaving a 30-kDa membrane-bound carboxyl C-terminal protein [24].

Glypican-3 has important biological roles in tumor development and progression. GPC3 silencing mediated by siRNA resulted in a temporary inhibition of cell migration and invasion in HCC cells, while reducing proliferation and inducing apoptosis in CP-Hep cells [23]. It regulates the signaling pathway of various morphogens, including Wnts signaling [25,26], interaction with fibroblast growth factors (FGFs) [27]and Hedgehogs (Hhs) [28], bone morphogenic proteins (BMPs) [29], promotion of Epithelial–Mesenchymal Transition (EMT) [30-32]. Moreover, GPC3 also plays a pivotal role in IGF-signaling pathway that influences cell proliferation [33], modulates G1 cell cycle progression [34], initiates and maintains oncogenesis [35], prevents apoptosis [36]. In a study, Wei Cheng etc found GPC3 decreased IGF-1-induced IGF-1R ubiquitination and degradation through the interaction between GPC3 and Grb10 [37].

It was found that GPC3 messenger RNA levels are significantly increased in most HCCs compared with nontumor livers [21,38]. A analysis from the Cancer Genome Atlas with the Broad Institute Firebrowse Gene expression viewer (http://firebrowse.org) found 61-fold increase in GPC3 expression in 373 HCC patients compared with 50 healthy individuals [39]. Hsu, H. C., et al. found that GPC3 is overexpressed in HCC and is associated with high alpha fetoprotein, high tumor grade, and high tumor aggressiveness [21]. Later, Hanlin L. Wang etc found that GPC3 is a specifc biomarker for HCC at the protein level(immunohistochemical staining), which can be used to distinguish HCC from benign hepatocellular diseases, particularly hepatocellular adenoma [40].

GPC3 can also be detected in the serum as a secreted protein of patients with HCC while it is undetectable in healthy individuals or patients with hepatitis or cirrhosis [41,42]. A meta-analysis found the overall diagnostic sensitivity and specificity for Diagnostic accuracy for hepatocellular carcinoma using serum glypican-3 were 0.53 (95% CI: 0.49-0.57) and 0.77 (95% CI: 0.74-0.81), respectively [43], which indicates the value of using serum GPC3 level in HCC diagnosis. What's more, GPC3 can distinguish alpha-fetal protein (AFP)-negative HCC from benign hepatocellular mass lesions, particularly hepatocellular adenoma [40,44], and distinguish AFP-negative benign hepatobiliary disease from normal controls [45], which indicates GPC3 a more reliable marker than AFP in HCC diagnosis and prevention.

GPC3 is used as a novel, targeted, and effective therapeutic options in immunotherapy for liver cancer, including GPC3-targeted reconstruction-antibody-based treatment [46,47], GPC3-derived peptide vaccine therapy [48,49], GPC3-targeted Gene therapies [39], recombinant immunotoxins [50]. Glypican-3 Redirecting T cells therapies [51-53], anti-GPC3 CAR-T Cells [54,55].

Early diagnosis

Glypican-3 (GPC3), a glycoprotein of glypican family that is overexpressed on the cell surface of Hepatocellular Carcinoma (HCC), is a promising biomarker for liver cancer diagnosis, and is an emerging candidate for novel molecular target therapies. Identification of patients with high GPC3 expression levels in HCC cells may have potential applications for the management of GPC3-targeting therapies.

Antibodies

Tissue GPC3 protein

As has been mentioned, GPC3 as a member of the glypican family attaches to the exocytoplasmic surface of the plasma membrane via a glycosyl-phosphatidylinositol anchor. Tissue/cell GPC3 levels in HCC patients can be measured using immunohistochemistry (IHC) and immunocytochemistry (ICC). Immunohistochemistry (IHC) involves a process of selectively imaging an antigen in a tissue section cell by utilizing an antibody that specifically binds to an antigen in a biological tissue [56]. IHC is the most familiar and readily available technique for locating biomarkers, and specific antibodies are now commercially available, reliable and suitable for paraffin-embedded and formalin-fixed tissue sections. IHC plays a crucial role in the diagnosis of hepatocellular carcinoma. In a study, Capurro, M., et al. report the detection of human GPC3 in paraffin-embedded tissues by the immunoperoxidase technique using anti-human GPC3 mouse monoclonal antibodies (mAbs). Routine histologic examination showed that GPC3 is expressed in 72% of HCCs (21 of 29), whereas it was not detected in a variety of other hepatic mass lesions and normal livers (9), including focal nodular hyperplasia (4), hepatocellular adenomas (7), low-grade dysplastic nodules (7), and hilar cholangiocarcinoma [22]. Another large number of hepatocellular mass lesions (221) were immunohistochemically examined using a monoclonal antibody specific for GPC3 to evaluate the diagnostic value of GPC3. Examination of cytoplasmic, membranous, and canalicular staining showed that GPC3 was expressed in 75.7% of HCCs (84 of 111), among which, 72.6% of cases (61 of 84) exhibited diffuse immunoreactivity, whereas it was not detected in a variety of 110 cases of hepatic mass lesions including hepatocellular adenoma, focal nodular hyperplasia, and large regenerative nodule [57]. Di Tommaso, L., et al. investigated the diagnostic value of 3 recognized putative markers of malignancy: heat-shock protein 70(HSP70), glypican 3 (GPC3), and Glutamine Synthetase (GS). Immunocytochemistry was used to detect these antigens. The sensitivity and specificity of the single tumor marker for the detection of early and grade 1 HCC (eHCC-G1) were 78% and 95% for HSP70, 69% and 91% for GPC3, and 59% and 86% for GS. Their study was also undertaken to investigate the diagnostic yield of a panel composed of the 3 markers, and also investigated the diagnostic yield of the composition of only 2 of the markers for eHCC-G1 detection [58] (see the table below).

Table 1:

Phenotype	eHCC-G1 (n = 32)	HGDN* (n = 22)	Sensitivity	Specificity	PPV	NPV
			3 Markers			
All 3 positive	14	0	43.75%	100%	100%	55.00%
At least 2 positive	23	0	71.88%	100%	100%	70.97%
At least 1 positive	29	6 (27.27%)	90.63%	72.73%	82.86%	84.21%
			2 Markers			
HSP70+/GS+	17	0	53.13%	100%	100%	59.46%
HSP70+/GPC3+	19	0	59.38%	100%	100%	62.86%
GPC3+/GS+	15	0	46.88%	100%	100%	56.41%
			1 Marker			
HSP70+	25	1 (4.54%)	78.13%	95.45%	96.15%	75.00%
GPC3+	22	2 (9.09%)	68.75%	90.91%	91.67%	66.67%
GS+	19	3 (13.64%)	59.38%	86.36%	86.36%	59.38%

Immunocytochemistry (ICC) is another common laboratory technique for anatomically visualizing the localization of a particular protein or antigen in a cell by utilizing an antibody that specifically binds to the antigen. Saverio Ligato et al. evaluated the immunocytochemical expression of GPC3 in the fine needle aspiration of hepatic lesions. 7 adenomas, 1 focal nodular hyperplasia (FNH), 24 HCCs, and 17 metastatic tumors were cytologically diagnosed. On the basis of the histological, clinical and/or radiological follow-up, 83.3% HCCs lesions (20/24) confirmed positive for GPC3. None of the seven adenomas and the only FNH were detected for GPC3. 16 of 17 metastatic malignancies were negative for GPC3, and the only one that expressed GPC3 was an anaplastic carcinoma with neuroendocrine features of unknown origin [59].

Serum levels of GPC3 protein

GPC3 can be cleaved by furin to generate a 40-kDa amino N-terminal protein, and the N-terminal protein can be cleaved from GPI anchoring site from the outer surface of the cell membrane and enter the bloodstream. The GPC3 level in the bloodstream of HCC patients can be measured by biochemical methods, such us Enzyme-Linked Immunosorbent Assay (ELISA) and sandwich Chemiluminescence Immunoassay (CLIA) [60-62]. To date, conflicting results for serum glypican-3 (GPC3) were reported in Hepatocellular Carcinoma (HCC) diagnosis. Many studies revealed that serum GPC3 is a specific biomarker for liver cancer diagnosis owing to its higher levels in HCC patients' serum level than that in healthy individuals or patients with hepatitis or cirrhosis [61,63-68]. GPC3 was also recommended as the biomarker for the diagnosis and treatment of HCC by the European Association for the Study of the Liver (EASL) and the American Association for the Study of Liver Disease (AASL). However, there are many negative cases suggesting that GPC3 alone may be not a promising tumor serum biomarker for either HCC [42,45,69,70] or Hepatoblastoma (HB) [45]. These variations may be due to the antibodies used for different epitopes, diversity of the samples, different detection methods, and data analysis. GPC3 may be not a prefect serum maker for the diagnosis of liver cancer alone, but it can be used in conjunction with other biomarkers or methods to diagnose liver cancer and elevate the sensitivity and specificity of diagnosis. Attallah AM et al. developed a GPC-HCC model for diagnosing HCC aimed to improve the diagnostic power of GPC3 by the combination of GPC3 with other laboratory simple routine tests. GPC-HCC model showed high HCC diagnostic accuracy in contrast to GPC3 and alpha fetoprotein (AFP) with area under the curve (AUC) of 0.939, sensitivity 93%, specificity 93%, positive predictive value 89%, negative predictive value 95%, and efficiency 93% [71]. Moreover, combination of GPC3 with one or more established biomarkers could elevate the sensitivity and specificity of liver cancer diagnosis [42,72].

Circulating tumor cells (CTCs)

The detection of circulating tumor cells (CTCs) which disengaged from the primary lesion and invade into the blood circulation can be a very powerful noninvasive approach for the early detection and therapy of liver cancer. The detection of CTCs in peripheral blood is called "a real-time liquid biopsy" with high efficiency, Sensitivity and specificity [73]. One problem is there are extremely rare cells making the capture and identification of CTCs a real challenge. Yuanfeng Pang, et al. used Surface-Enhanced Raman scattering (SERS) spectroscopy analytical technique for biological sensing and trace analysis, and in the meantime used a biosensor which consists of two basic elements including an anti-ASGPR antibody-Fe_3O_4@Ag MNPs and an anti-GPC3 antibody Au@Ag@DTNB NRs. The dual-enhancement SERS and dual-selective nanospheres system can detect HCC CTCs for rapid, efficient capture and sensitive with a good linear relationship (R2 = 0.99) between the SERS intensity and the concentration of HCC CTCS in the range of 1-150 cells/mL can be observed with a LOD of 1 cells/Ml [74]. A research present an efficient, practicable cell sorting strategy based on anti-CD45 antibody modified magnetic nanospheres to purify the spiked HCC cells, and with specific identification biomarkers AFP and GPC3, effective isolation of HCC cells can be achieved within 30 min, with a huge number of magnetically labeled leukocytes depleted from the mixed peripheral blood samples by external magnetic field [75]. The monitoring of circulating tumor cells (CTCs) is a very beneficial tool in diagnosis, predicting chemotherapy response and even disease progression. Furthermore, provided with more targeted tumor markers, CTCs can be used in the automatic detection of multi-biochemical indicators.

Glypican-3-binding peptide

Short peptide is a promising molecular imaging probes that have been rapidly evolving due to the advantages of low molecular weight, easey to modification and scale-up [76]. With multiple high-throughput technologies, identification and affinity maturation will be more accurate and faster, while overall optimizing the process of translating novel ligands into clinical [77]. Dongling Zhu et al. identified a 12-mer peptide with the

sequence of DHLASLWWGTEL (denoted as TJ12P1) by screening a phage display peptide library that demonstrated ideal GPC3 binding affinity. TJ12P1 conjugated with nearinfrared fluorescent (NIFR) dye Cy5.5 has significantly higher tumor accumulation in xenografts of HepG2 mice, making it a promising GPC3-binding peptide ligand for detecting the GPC3 expression in HCC not only in vitro but also in vivo by its non-invasive imaging [76].

Hong-Yang Wang et al. developed a water soluble, biocompatible 2D material-based supramolecular imaging probe using a known peptide ligand (RLNVGGTYFLTTRQ) [78], which can sensitively and selectively image cells overexpressing GPC-3. It can effectively detect malignancy in frozen sections in a rapid and precise manner and guide the subsequent surgical program during the surgery. Their research offers new insights into the precise and effective clinical diagnosis of HCC by using peptide ligand [79]. The same peptide ligand (RLNVGGTYFLTTRQ) was used by Wang Z et al. to develop a novel PET probe for imaging the expression of GPC-3 to achieve a high enough tumor/liver ratio for detection of the tumor in the liver [80].

Aptamer

Aptamers are a single-stranded RNA or DNA (ssDNA) oligonucleotide with secondary and tertiary structures with highly specific for certain targets. They have similarity to protein antibodies that bind to a specific target molecule, but they also have many unique properties in biomarker discovery, in vitro and in vivo diagnosis, precisely controlled drug release, and targeted therapy. Aptamers can almost avoid immunogenicity and toxicty in vivo to reduce unforeseen side effects. Due to its small size, the aptamer can strongly penetrate into the tissue and be easily internalized by its target cells, thereby improving the ratio of tumor to blood and tumor to normal tissue and enhancing its therapeutic index. They are more stable and can be stored and transported easily [81].

A study introduced a novel GPC3 specific aptamer (AP613-1) which displays a specific binding affinity to GPC3 positive HCC. AP613-1 labeled with Alexa Fluor 750 (AF750) could specifically bind to GPC3 and could be revealed by the NIR fluorescence imaging. The fluorescence intensities were in excellent correlation ($P<0.001$, $r=0.968$) [82]. Due to the specific binding affinity and inherent advantages, aptamers may play an important role in clinical and experimental studies, such as intraoperative HCC visualization to assess tumor invasion during operation, which can help surgeons to precisely remove the malignant tissues.

Other methods

GPC3 messenger RNA levels are significantly increased in most HCCs compared with nontumor livers [38]. Unlike protein, mRNAs are much more stable than plasma proteins. Xie H et al. built a non-invasive, accurate, and fast method for early detection of HCC to analyze genes expression in the peripheral blood samples by different multi-parameter analysis methods and build a diagnostic model to classify hepatocellular carcinoma patients and healthy people. In the study, Affymetrix was used to screen differential gene expression, and they built a 9-gene(GPC3, HGF, ANXA1, FOS, SPAG9, HSPA1B, CXCR4, PFN1, and CALR) expression detection system based on the GenomeLab GeXP Genetic Analysis system. The sensitivity and specifcity of the 52 HCC patients and 34 healthy normal controls were 96% and 86% respectively [83].

Another study present a promising new method to detected GPC-3 mRNA together with AFP mRNA in extracellular vesicles (EVs) in patient plasma using designed molecular beacons and a novel tethered lipoplex nanoparticle (TLN) biochip. The detection of EV AFP and GPC-3 mRNAs provided an AUC (area under the ROC curve) of 0.995, better than that of a single marker [84]. This method can detect smaller fragmented transcripts using fewer samples compared to Quantitative Reverse Transcriptase PCR (qRT-PCR). Their new method has a potential value for risk stratification in liver cancer screening, therapeutic monitoring, and after-treatment surveillance.

Target therapy

Liver cancer is one of the highest causes of cancer-related deaths worldwide due to a lack of early detection strategies and effective treatment. Multikinase inhibitors (sorafenib/regorafenib) are the two first line Chemotherapeutics approved by U.S. Food and Drug Administration (FDA) for advanced liver cancer treatment with limited treatment effect. Sorafenib the first targeted drug approved by FDA for advanced liver cancer treatment is a multi-tyrosine kinase inhibitor that targets the activity of Ras/MAPK signaling (b-RAF), Vascular Endothelial Growth Factor (VEGF) receptor and platelet-derived growth factor (PDGF) receptor to influence tumor cell proliferation and angiogenesis [85,86]. It was over 10 years since FDA first approved Sorafenib for the dvanced liver cancer treatment. A study covered 1,532 patients were carried out to evaluate the effectiveness of initial sorafenib versus no treatment among Medicare beneficiaries with advanced HCC. However, the survival rate after starting sorafenib treatment in newly diagnosed Medicare beneficiaries with HCC is very short, indicating that Sorafenib is not applicable to all HCC patients [87]. Beside of the limited benefit, drug-related symptom burden and high drug cost-must be considered. Another study found Sorafenib is associated with improved survival in elderly patients with advanced HCC; but, it is not cost-effective among those with hepatic decompensation [88]. In April 2017, regorafenib (Stivarga), an oral diphenylurea multikinase inhibitor that targets angiogenic (VEGFR1-3, TIE2), stromal (PDGFR-β, FGFR), and oncogenic receptor tyrosine kinases (KIT, RET, and RAF) was approved by FDA to systemically treat patients with HCC who progressed after sorafenib treatment [89]. On September 22, 2017, an immunotherapy drug nivolumab (Opdivo®) was accelerated approved by FDA for some patients with advanced liver cancer (hepatocellular carcinoma). Of the three FDA approved drugs for liver cancer treatment only the latter having potential to dramatically improve outcome, emphasizing the effectiveness for immunotherapy which may change the landscape of oncology care in the future. In this part, various immunotherapies targeting GPC3 will be discussed to show possible ways of target therapies for liver cancer.

Antibody-based therapeutics

Antibody drug conjugates (ADC)

As said above, there are many side effects of Sorafenib (SFB) although it has improved the treatment of hepatocellular carcinoma (HCC). Reasons may include lacking of tumor-specific targeting, short half-life in vivo [90] as well as drug resistance. To increase tumor-specific targeting and permanently maintain concentration of the drug in the tumor tissue at a level that inhibits tumor growth. Tang, X., et al. developed a novel SFB-loaded polymer nanoparticle (NP) which contains PluronicP123 and SFB, and its surface is modified with anti-GPC3 antibody(Ab) to produce the polymer nanoparticle (NP-SFB-Ab). The NP-SFB-Ab displayed higher cellular uptake by HepG2 cells, released higher

levels of SFB into cell culture medium, showed better stability characteristics, had a promising toxicological profile and was more cytotoxic to HCC cells than was non-targeted NP-SFB and free SFB. What's more, in vivo studies of HepG2 xenograft tumors in nude mice confirmed that NP-SFB-Ab inhibited tumor growth to a greater extent than did NP-SFB or free SFB without producing obvious side effects. The confirmed mechanisms are downregulating MEK 1/2 and ERK phosphorylation by inhibiting Raf kinase, and inhibition of RAF/MEK/ERK signaling pathway. NP-SFB-Ab also downregulated Mcl-1 expression to promote the release mitochondrial cytochrome C, and thereby induces apoptosis by polymerizing Bax and Bak on the mitochondrial membrane [46]. Similarly, another group also developed a novel SFB-loaded polymeric nanoparticle (NP-SFB-Ab) for targeted therapy of liver cancer. The NP-SFB-Ab displayed higher cellular uptake by HepG2 human liver cells, released excellent levels of SFB into cell culture medium, showed robust stability characteristics, caused much greater cytotoxicity than non-targeted NP-SFB and free SFB. What's more, in vivo studies of HepG2 xenograft tumors in nude mice confirmed that NP-SFB-Ab inhibited tumor growth to a greater extent than did NP-SFB or free SFB without producing obvious side effects [91]. Therefore, antibody drug conjugates (ADC) would be a novel promising medicine platform for targeted liver cancer therapy.

Bispecific antibody

Bispecific T Cell–Redirecting Antibody (TRAB) is a promising immunotherapy, which can redirect T cells to tumor cells by engaging CD3 on a T cell and an antigen on a tumor cell regardless of the specificity of T cell receptors, and is considered efficacious for tumors lacking enough neoantigens. The technology requires proteins expressed almost exclusively in tumors to reduce the likelihood of on-target, off-tumour toxicity. GPC3 is a highly tumor-specific antigen as we can see from mRNA expression data and protein level examination by immunohistochemistry. Ishiguro T, et al. generated a fully humanized immunoglobulin G (IgG)–based bispecific antibody: an anti-GPC3 TRAB, ERY974. ERY974 was generated by combining two antibodies using an antiGPC3 antibody (clone GC33) and an anti-CD3 antibody (clone CE115), which engage CD3 to direct T cells to the target antigen on solid tumors. Potency of ERY974 in GPC3-positive cell lines and in xenograft models that were injected with human T cells were investigated. The results showed that ERY974 not only has highly effective killing effect on various tumors with GPC3 expression in clinical tumors, but also induced a robust antitumor efficacy even against tumors with non-immunogenic characteristics which are difficult to treat by inhibiting immune checkpoints such as PD-1 (programmed cell death protein-1) and CTLA-4 (cytotoxic T lymphocyte-associated protein-4). Treatment of cynomolgus monkeys with ERY974 showed transient blood cytokine elevation but premedication with corticosteroids almost completely inhibited cytokine release, whereas it did not affect the antitumor efficacy [47].

Wang Y, et al. constructed a bispecific antibody (bsAb), GPC3-S-Fab designed by linking the Fab of anti-GPC3 antibody GC33 with an anti-CD16 single domain antibody. Their study found that purified GPC3-S-Fab can recruit NK cells targeting GPC3 positive tumor cells suggesting a potential application of GPC3-S-Fab in liver cancer therapy. What's more, their antibody can easily be produced in the periplasm of bacteria and purified by a two-step affinity purification on a large scale [92].

To accurately show different kinds of bispecific antibodies, we recommend you the comprehensive review written by Fan GW that summarized diverse formats of bsAbs and their clinical applications and clarified strategies for optimizing bsAbs design [93].

Chimeric antigen receptor T cells (CAR-T)

CAR-T cell therapy is one of the immunotherapies by expanding T cells ex vivo and modified them with a chimeric antigen receptor (CAR) before infusing them back into patients. It can concentrate tumour-specific CTLs in the tumour microenvironment and has achieved significant clinical success [53]. Biomaterials for the in vitro expansion of CAR T cells and enable controlled release of immunomodulatory agents have been well established [94]. In recent years, CD19-specific CAR-T cells have generated impressive results in patients with B-cell lymphoid malignancies [95-97]. However, the response rate to CAR T therapy in the early clinical testing of various solid tumors is still low [98,99]. GPC3 as an attractive target for immunotherapy can be a novel antigens to optimize antigen-specific CAR design. Jiang Z, et al. built a general clinic-relevant model for hepatocellular carcinoma (HCC) to evaluate the cytotoxicity of adoptive chimeric antigen receptor (CAR) T cells (GPC3-CAR T cells) used in HCC. Results found GPC3-CAR T cells can effectively eliminate tumors in patient-derived xenograft models of HCC, demonstrating that GPC3-CAR T cell therapy is a promising candidate for HCC therapy [54].

In a study Li W, et al. systematically evaluated a series of CAR constructs targeting GPC3 and compared the relative contribution of GPC3-specific CARs that encoded CD3f (Gz) alone or with co-stimulatory domains derived from CD28 (G28z), 4-1BB (GBBz), or CD28 and 4-1BB (G28BBz). They found all constructed GPC3-CARs modified T cells had highly cytotoxicity to GPC3-positive hepatocellular carcinoma, hepatoblastoma, and malignant rhabdoid tumor cell lines in vitro and induced sustained tumor regressions in GPC3-positive tumor xenografts [52].

To solve the intrinsic inhibitory pathways mediated by upregulated inhibitory receptors reacting with their cognate ligands in the tumor microenvironment and increase efficacy of GPC3 CAR-T cells with solid tumors, Pan, Z., et al. introduced a GPC3-specifc CAR-T cells carrying sPD1(a fusion protein composed of a PD-1 extracellular domain and CH3 from IgG4) to block the PD-1/PD-L1 pathway [100].

Similarly and increasingly, Rafiq S, et al. modified CAR-T cells to secrete PD-1-blocking single-chain variable fragments (scFv). Their modified CAR-T cells can secret anti-PD-1 scFv in both a paracrine and autocrine manner that can improve the anti-tumor activity of CAR-T cells and bystander tumor-specific T cells in clinically relevant syngeneic and xenogeneic mouse models of PD-L1+ hematologic and solid tumors. The efficacy was even better than the combination therapy with CAR-T cells and a checkpoint inhibitor [101].

Recombinant immunotoxins (RITs)

Recombinant immunotoxins (RITs) are chimeric proteins composed of a specific antibody fragment fused to a toxin with high potency that kills tumor cells. Fragments of different antibodies direct the toxin to cancer cells that express the internalized target antigen. RITs have an advantage over standard chemotherapies to induce potent cytotoxicity even in cancer cells known to be resistant to standard chemotherapy and to reduce off-target side effects [102]. To date, many immunotoxins are currently in preclinical development to target cancer. The most effective immunotoxins for treating human cancer are CD22 im-

munotoxins for hairy cell leukaemia and other CD22-positive leukaemia cells [103-105]. GPC3 as a specific biomarker for HCCs can be an ideal target for immunotoxins. Pseudomonas exotoxin A (PE38) is a favorable toxin for construction of RITs because of its high cell killing activity and sites for endurable mutations [106]. A group generated human monoclonal antibody VH domain (HN3) targeting a conformational epitope in GPC3 that inhibits Yap signaling and are fused to a fragment of PE38 to create immunotoxins (HN3-PE38). Intravenous injection of HN3-PE38 alone or in combination with chemotherapy induced regression of liver tumour xenografts in mice through inactivation of Wnt-induced signalling via the HN3 antibody fragment and the cell killing activity via PE38 [107]. However, the clinical success of RIT in patients with normal immune systems is limited by its immunogenicity with induced neutralizing antibodies in humans. Ways can be found to reduce the immunogenicity by balancing act between immunogenicity and therapeutic potency [108]. Later, to avoid off-target toxicity and induced neutralizing antibodies in humans of PE38, the same group engineered a second generation PE fragment (mPE24). They constructed the monovalent HN3-mPE24 immunotoxin (39 kDa) using the engineered toxin fragment mPE24, and found HN3-mPE24 immunotoxin lead to a dramatic decrease in tumor volume and prolonged the survival rate of the mice, and had no obvious side effects [109].

Gene therapies

Gene therapy or human gene transfer involves precise delivery of therapeutic nucleic acid into a patient's cells. Gene therapies are the promising therapeutic avenues under investigation for the past years but are not largely explored for HCC. Gene expression alteration targeting RNA is a common Gene therapy application for liver cancer including the use of miRNAs, short hairpin RNAs (shRNAs), and siRNAs had certain efficacy in the GPC3 target Gene Therapies of HCC models [110-113]. Recently, Dhungel et al. focused on transcriptional differences between HCC and hepatocytes and examined the potential of the GPC3 promoter as a targeting strategy. They found GPC3 promoter can drive more luciferase and eYFP expression in HCC cell lines compared to normal hepatocytes. What's more, they constructed a vector containing cytosine deaminase (CD) under the control of GPC3 promoter, which showed significantly more killing efficacy to HCC cell lines compared to normal liver cell lines after treatment with 5-FC. Their study suggest that transcriptionally targeted delivery of therapeutic nucleic acid into HCC cells can be achieved using the GPC3 promoter and in the meantime produces limited toxicity to normal liver cells [39].

Glypican-3 vaccines

A Japanese group thinks adoptive immunotherapy with T cells transduced with TCRs obtained from CTLs generated by cancer peptide vaccines can achieve better antitumor effects. They developed an array of GPC3-based peptide vaccines from the last 17 years and that have now been tested clinically as cancer vaccines. They carried out a phase I clinical trial of GPC3 peptide vaccines (UMIN Clinical Trials Registry: 000001395 2007.02~2009.11). Result showed GPC3 vaccination was well tolerated and the peptide vaccine induced a GPC3-specific CTL response in 91% patients (30/33) [114]. A subsequent phase I trial (UMIN Clinical Trials Registry: 000005093) found vaccine that induced GPC3 peptide-specific CTLs was found to infiltrate into the tumor, but most of these patients were nearly unresponsive to the vaccine. The underlying reason they explained is the patients with late-stage cancer after sorafenib as chemotherapy were unsuitable for the vaccine therapy [49]. A single-arm phase II clinical trial (UMIN Clinical Trials Registry: 000002614) using GPC3 peptide vaccine as adjuvant therapy to evaluate 1- and 2-year recurrence rates in 41 patients following radical treatment of HCC showed 24.4% and 53.7% for the 1- and 2-y recurrence rates respectively with the primary endpoint unreached. Two cases of relapse with GPC3 that was expressed in the primary tumor, but not in the recurrent tumor suggest that if the tumor does not express or lose the same antigen, proliferation may occur [115]. So they highlight the importance of vaccines that target multiple tumorassociated antigens. Later, they carried out preclinical research on intratumor injection [116] and combination therapy with immune inhibitor PD-1 [117], CD4 [118] and other immunomodulators. These researches showed that such measures can enhance the efficacy of peptide vaccines.

Chen K, et al prepared a GPC3 nanovaccine incorporating a Toll-like receptor (TLR)-7/8 agonistCL097 as adjuvant using Synthesizing mannosylated liposomes (LPMan) as vaccine delivery system (LPMan-GPC3/CL097). Immunotherapy with the nanovaccine generated significantly more GPC3-specific CD4+ IFNγ- and CD8+ IFNγ-producing T cells in mice spleens and livers, and prevented HCC development [119].

Conclusion

Overall, the past recent years of GPC-3 studies have laid the foundation for precise personalized diagnosis and targeted therapy in liver cancer. GPC3-targeted imaging in vivo or in vitro of the liver tissues and serum will emerge as a viable option in the year to come in the prevention, pretreatment, operation, monitor, and prognosis of liver cancer. Conflict results in the serum GPC3-targeted diagonosis and the emerged solution showed us a new dimension in joint diagnosis using different biomarkers such as AFP, heat-shock protein 70(HSP70), Glutamine Synthetase (GS), Arginase-1, and/or diferrent methods such as Ultrasonography (US), Computed Tomography (CT), high-cost Magnetic Resonance Imaging (MRI) and other simple routine laboratory tests. Preclinical and clinical therapeutic researches obtained so far give us an optimistic view about GPC3-targeting immunotherapies including ADC, TRAB, CAR-T, RITs, Gene Therapies, glypican-3 vaccines and so on. What's more, combination therapies of GPC3-targeting immunotherapies, chemotherapeutics, immune checkpoints inhibitors, or surgical resection and liver transplantation are promising approach for successful treatment of liver cancer. Further studies to thoroughly understand of the complex mechanism underlying GPC3 involvement in liver progression are crucial. We now look forward to future developments on GPC3 to bring clinical benefits to patients with liver cancer.

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