Glypican-3 (GPC3) is a member of the glypican family. Glypicans are proteoglycans that are attached to the cell surface by a glycosyl-phosphatidyl-inositol anchor. They regulate the signaling activity of several growth factors, including Wnts. This regulation is based on the ability of glypicans to stimulate or inhibit the interaction of these growth factors with their respective signaling receptors. It has been clearly established that whereas GPC3 is expressed by most hepatocellular carcinomas (HCCs), this glypican is not detected in normal and cirrhotic liver, or in benign hepatic lesions. Consequently, immunostaining of liver biopsies for GPC3 is currently being used by clinical pathologists to confirm HCC diagnosis when the malignant nature of the lesion is difficult to establish. In addition to being a marker of HCC, GPC3 plays a role in the progression of the disease. GPC3 promotes the growth of HCC by stimulating canonical Wnt signaling. It has been proposed that this stimulation is based on the ability of GPC3 to increase the binding of Wnt to its signaling receptor, Frizzled. Two therapeutic approaches for HCC that target GPC3 are currently being tested in phase II clinical trials. One of them is based on the use of a humanized GPC3 monoclonal antibody that inhibits the in vivo growth of HCC xenografts by inducing antibody-dependent cellular cytotoxicity. The second approach employs a vaccine that consists of two GPC3-derived peptides that induce cytotoxic T lymphocytes against these peptides. Targeting of GPC3 might offer a new tool for the treatment of HCC.
respective signaling receptors [1]. The specific function of a glypican in a particular cell context depends on its structural features and on the set of growth factors and growth factor receptors present in that cellular context.

**Hepatocellular carcinoma (HCC)**

HCC is the sixth most common cancer in the world, and the third most frequent cause of cancer-related death [14]. Individuals with cirrhosis are at high risk of developing this disease. In general, potentially curative therapeutic approaches are available only for cases in which the diagnosis is done at an asymptomatic early stage [15]. Advances in imaging techniques and the establishment of surveillance protocols for individuals at high risk has led to the detection of small hepatic nodules that could represent benign or malignant lesions but definitive diagnosis requires histological examination of biopsy material [16]. However, in many cases, conventional pathological analysis of these biopsies is very difficult, and the availability of one or more tumor markers that could confirm the malignant nature of the lesion could significantly improve diagnosis accuracy [17].

**GPC3 as a marker for HCC**

In 1997, Hsu et al. reported that GPC3 mRNA levels are significantly elevated in most HCCs compared with normal liver and non-malignant liver lesions [18]. Significantly, GPC3 mRNA levels were more frequently elevated in HCC than those of alphafetoprotein (AFP), a commonly used marker of HCC (88% versus 55%), and the difference was even greater in HCCs smaller than 3 cm (77% versus 43%). These results were later confirmed at the protein level [19]. By performing immunohistochemistry with a monoclonal antibody, Capurro et al. [19] found that 72% of HCCs express GPC3, whereas this protein was not detected in normal hepatocytes, cirrhotic liver or benign lesions. Staining was localized at the cell membrane and/or the cytoplasm. Similar results were obtained later by many laboratories around the world [20–23]. Some of these studies included thin-core biopsy specimens and early stage HCCs [24–26]. Consequently, a report of the International Consensus Group for HCC on the pathologic diagnosis of early HCC stated that ‘GPC3 immunoreactivity has a reported sensitivity of 77% and specificity of 96% in the diagnosis of small HCC; therefore, GPC3 positivity is a strong argument for malignancy’ [27]. Because in a significant number of HCCs only a proportion of the malignant cells express GPC3, the sensitivity of the immunostaining could be lower when tissue microarrays with small tissue sections are used [28].

Many gastrointestinal and pancreatic tumors tend to form metastases in the liver. Mounajjed et al. recently investigated whether GPC3 immunostaining could be used to distinguish these metastases from primary HCCs. They found that 14% of the metastases from gastrointestinal and pancreatic tumors are GPC3-positive, suggesting that this marker is not very specific in distinguishing HCC from metastases originating from extra-hepatic sources [29].

HCCs are highly heterogeneous, and it is unlikely that a single marker will be found that is expressed in 100% of patients [30]. Di Tommaso et al. have shown that the simultaneous use of GPC3, heat shock protein 70 (HSP70) and glutamine synthetase (GS) immunostaining could significantly increase sensitivity without reducing specificity of the GPC3-only based diagnosis of HCC [31,32]. Their conclusions were confirmed in a more recent prospective study [33]. The American Association for the Study of Liver Disease has stated in a recent update on the management of HCC that ‘Expert pathology diagnosis is reinforced by staining for GPC3, HSP70 and GS, because positivity for two of these three stains confirms HCC’ [34]. Likewise, the Clinical Practice Guidelines of the European Association for the Study of the Liver also recommend the use of this panel of markers to confirm HCC diagnosis [35].

Multivariate analysis showed that GPC3 expression is an independent predictor of poor prognosis in HCC patients regardless of etiology [36]. However, another study found that this correlation was only valid for hepatitis virus C patients with clear cell surface staining [37]. Additional work will be required to solve this controversy.

Early diagnosis of HCC would be much easier if one could identify a marker that specifically appears in the serum of patients with small HCCs. An initial study by Capurro et al. reported that, whereas GPC3 could be found in the serum of 53% of HCC patients, it was undetectable in normal serum [19]. In addition, these authors reported that only 1 of 20 patients with hepatitis and cirrhosis was GPC3-positive [19]. Similar results were obtained by two other laboratories [38,39]. Clearly, more extensive studies including a much larger number of patients will be required to confirm these initial findings.

**GPC3 promotes the growth of HCC**

In addition to being a marker of HCC, GPC3 plays a role in the progression of the disease [40]. By studying the effect of ectopic GPC3 in various HCC cell lines,
Capurro et al. [40] have shown that this glypican promotes the in vivo and in vitro growth of HCC by stimulating canonical Wnt signaling. The activation of this signaling pathway induces the cytosolic accumulation and nuclear translocation of the transcription factor β-catenin. In the nucleus β-catenin associates with members of the LEF/TCF family of transcription factors, and induces the expression of genes that stimulate cell cycle progression and cell survival [41]. Consequently, canonical Wnt activity has been shown to play a role in the progression of many cancer types, including HCC [41,42]. In colorectal cancer, for example, canonical Wnt signaling is activated in over 90% of patients by mutations in the APC and β-catenin genes [43]. In HCC, however, mutations in these genes are very rare, despite the fact that canonical Wnt signaling, as assessed by the cytoplasmic and nuclear accumulation of β-catenin, is frequent [44,45]. Thus GPC3 overexpression represents an alternative mechanism by which Wnt activity can be stimulated in HCC. This GPC3-induced activation of canonical Wnt signaling in HCC cells has recently been confirmed by Li et al. [46]. These authors showed that ectopic GPC3 in HCC cells induces the expression of c-Myc, a well-characterized target of canonical Wnt signaling. The GPC3-induced expression of c-Myc could be blocked by downregulating β-catenin [46].

Several reports have provided some clues with regard to the mechanism by which GPC3 stimulates Wnt signaling. First, it has been shown that GPC3 can form a complex with several Wnts [40]. Because Wnts are heparin-binding molecules, it was expected that complex formation would require the HS chains of this glypican. Surprisingly, however, it has been shown that Wnt3a and Wnt7b can form a complex with a GPC3 mutant that does not have HS chains [40]. Consistent with this observation, this GPC3 mutant is able to partially stimulate canonical Wnt signaling in vitro and in vivo in most cell types [40]. Similar results were found in Drosophila, were a non-glycanated Dally-like protein (one of the two Drosophila glypicans) was shown to stimulate canonical Wnt signaling and co-immunoprecipitate with Wingless (a Drosophila Wnt) [47]. GPC3 is normally cleaved by a convertase into two subunits [48]. Interestingly, it has been reported that this cleavage is not required for the GPC3-induced stimulation of canonical Wnt signaling [49]. On the other hand, attachment to the cell membrane is essential for this GPC3 activity [40].

In addition to forming complexes with Wnts, GPC3 can interact with various Frizzleds, the signaling receptors for Wnts (M. Capurro and J. Filmus, unpublished observations). Based on this, we have proposed that GPC3 stimulates canonical Wnt signaling by forming a complex with Wnt and Frizzled and facilitating/stabilizing the binding of these two proteins (Fig. 1) [1].

Although, as described above, the HS chains are not required for the interaction of GPC3 with Wnts, at least in some cellular contexts these chains are required for optimal GPC3-induced activation of Wnt activity [40]. The molecular basis for this cell-type-specific role of HS remains unknown.

It is also possible that in certain HCCs GPC3 promotes tumor growth by activating other signaling pathways. For example, it has been reported that the FGF signaling pathway is activated in a significant proportion of HCC tumors [50], and it is well established that glypicans can stimulate FGF activity [51].

**GPC3 as a target for HCC treatment**

There is an urgent need for novel targeted treatments for advanced HCC [52,53]. The fact that GPC3 is expressed by malignant liver cells but not by normal or cirrhotic liver suggests that such novel therapeutic approaches for HCC could be generated by targeting this protein.

One approach to targeting GPC3 in HCC that has shown significant progress is the use of GC33, a humanized anti-GPC3 monoclonal antibody [54]. Ishiguro et al. showed that this antibody significantly inhibits the growth of GPC3-positive human HCC xenografts in
SCID mice. GPC3-negative HCC xenografts, on the other hand, were not affected by this treatment [54]. These investigators also demonstrated that, as expected, the mechanism of the GC33-induced tumor growth inhibition is an antibody-dependent cellular cytotoxicity [54, 55]. Based on these results, a phase I clinical trial with patients with advanced HCC has recently been performed. It was found that GC33 can be safely administered intravenously up to 20 mg kg⁻¹ weekly [56]. Consequently, patients with advanced metastatic HCC are currently being recruited for a phase II clinical trial (www.clinicaltrials.gov/ct2/show/study/NCT01507168).

Nakatsura and colleagues have identified a GPC3 peptide vaccine that induces peptide-reactive cytotoxic T lymphocytes (CTLs) in HLA-A2.1 transgenic mice without producing autoimmunity [57]. Furthermore, they showed that the inoculation of these CTLs significantly inhibits the growth of human HCC xenografts in NOD/SCID mice. On the basis of these results they have recently performed a phase I clinical trial of a vaccine composed of two GPC3-derived peptides and an incomplete Freund’s adjuvant in advanced HCC patients. They found that the vaccination was well tolerated and that it triggered a measurable immune response in 30 out of 33 patients [58]. Furthermore, they observed a correlation between the levels of the immune response and overall survival. The investigators are now proceeding with a phase II study where they also plan to combine the GPC3-derived peptide vaccine with chemotherapy [58].

In addition to targeting GPC3 by immunotherapeutic approaches, investigators have tried to inhibit HCC growth by blocking GPC3 function. Zittermann et al. [59] have recently shown that a mutant GPC3 that cannot be attached to the cell membrane (GPC3ΔGPI), and is therefore secreted to the extracellular environment, could remove Wnt from the cell surface and reduce its binding to Frizzled. Furthermore, these investigators showed that ectopic GPC3ΔGPI inhibits the in vitro and in vivo growth of several HCC cell lines. Significantly, Zittermann et al. showed that, in addition to inhibiting Wnt activity, in specific HCC cell lines GPC3ΔGPI inhibits signaling pathways that could be triggered by heparin-binding pro-tumorigenic growth factors such as FGF or hepatocyte growth factor, which are frequently expressed by HCC cells. Because GPC3 can bind through its HS chains to these growth factors, it was proposed that this inhibition was due to the fact that GPC3ΔGPI could also remove these factors from the surface [59]. More recently, another laboratory confirmed the growth-inhibitory activity of GPC3ΔGPI by treating HCC cells with recombinant protein [60].

**Conclusion**

The discovery by basic scientists that GPC3 is expressed by most HCCs and that it is not produced by normal or cirrhotic liver has already had a significant impact in diagnostic clinical practice. Ongoing clinical trials will establish in the future whether the impact of the initial discovery will also be extended to the therapy of HCC.

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