REVIEW ARTICLE

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Glypican-3 and Alphafetoprotein as Diagnostic Tests for Hepatocellular Carcinoma

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Contents

Abstract

Hepatocellular carcinoma (HCC) is one of the most common types of malignant tumor. It is usually asymptomatic in the early stages and tends to be intravascularly and intrabiliary invasive. Therefore, most patients present with incurable disease at the time of detection and early diagnosis of HCC is critical for a good prognosis.

The imaging-based diagnosis of small tumors is relatively inaccurate, as cirrhotic and dysplastic nodules mimic HCC radiologically. The availability of a suitable serological marker to distinguish between HCC and benign liver lesions would, therefore, be very useful for early diagnosis. The only serological marker currently widely used for the diagnosis of HCC is alphafetoprotein (AFP). However, the sensitivity of this marker is limited (41–65%). Given the high heterogeneity of HCC, it is currently thought that an optimal serological test for HCC will be based on the simultaneous measurement of two or three highly specific serological markers.

Several laboratories have recently reported that glypican-3 (GPC3), a membrane-bound proteoglycan, is expressed by a large proportion of HCCs, but is undetectable in normal hepatocytes and non-malignant liver disease. Furthermore, various studies demonstrated that GPC3 could be used as a serological test for the diagnosis of patients with HCC. Although the specificity of the test was very high in the context of a population with chronic liver disease, the sensitivity was limited (within the same range as AFP). Interestingly, in most cases, elevated GPC3 values did not correlate with elevated AFP values. As a consequence, the serological level of at least one of the two markers was elevated in a large majority of HCC patients. These results suggest that the sensitivity of the diagnostic test can be significantly improved without compromising specificity with the simultaneous measurement of both GPC3 and AFP.

1. Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is the fifth most common neoplasm in the world, and the third most common cause of cancer-related death.[1-4] Every year more than 500 000 new cases are diagnosed worldwide.[3,4] Although HCC is more common in Asia and Africa, its incidence has increased substantially in many Western countries during the last twenty years.[3,5,6] A recent study

comparing the molecular clock of hepatitis C virus in the US and Japan predicts that the burden of HCC in the US will reach that of Japan in the next two to three decades.[7]

HCC is usually asymptomatic in the early stages and has a tendency for intravascular or intrabiliary invasion, even when the primary tumor is small.[8] Thus, when HCC symptoms appear it is at an advanced stage. Early diagnosis is, therefore, critical for a good prognosis. Historically, only 10–20% of primary HCCs are found to be resectable at the time of diagnosis.[8] More recently, with the advent of HCC screening programs, the proportion of potentially curable tumors has increased, although the percentage is still small.

HCC is associated with chronic liver injury, primarily chronic viral hepatitis and alcoholic liver disease.[3,4,9] The highest incidence of HCC is found in areas where the hepatitis B (HBV) and hepatitis C virus (HCV) are endemic. In the case of HBV, it has been demonstrated that the relative risk of developing HCC is 25- to 35-fold greater in individuals with evidence of chronic infection compared to non-infected individuals.[10] In chronic HCV there are no estimates of relative risk, but the incidence of HCC in cirrhotic carriers of HCV may be as high as 5% per year, compared with 0.5% in HBV carriers.[11] Persistent HCV infection is the cause of 70% of HCC cases in Japan, and the most likely reason for the rising incidence of HCC in North America is the increased spread of HCV infection within the population.[12,13]

2. Diagnosis

Diagnosis of HCC is usually easy in patients with a space-occupying lesion on ultrasonography or computed tomography, and serological alphafetoprotein (AFP) of more than 400 ng/mL.[8] In many cases, however, by the time these conditions are met, the HCC is incurable, as frequently the AFP is not diagnostically elevated.

Diagnosis by the imaging of small lesions is relatively inaccurate, whether by ultrasonography, computed tomography scanning, or MRI.[8,14] In particular, the two benign lesions which may mimic HCC radiologically are cirrhotic and dysplastic nodules. Liver biopsy of small lesions is also insufficiently sensitive or specific.[15] Even with a needle biopsy, a well-differentiated cancer may be difficult to distinguish from a benign lesion due to the limited amount of material usually obtained by this procedure.[8] Thus, despite advances in imaging technology, there is still a need for suitable serological markers to distinguish between HCC and benign liver lesions in difficult cases.

3. Screening

One striking feature of HCC is that most of the risk factors are well known: at least 90% of cases occur in patients with chronic liver disease (CLD), and most also have cirrhosis.[16] Thus, a sensitive and specific serological marker for HCC would also be useful for the screening of patients at risk. For example, the risk for HCC in patients with cirrhosis is estimated to be 1–4% per year. This risk is clearly higher than the 0.2% per year at which the consensus suggest that surveillance is warranted.[17]

Although the screening of patients at risk of HCC is widely applied, as yet there is little data to suggest that it has been effective in reducing disease-specific mortality.[18] The most common screening protocol includes a serological AFP test and ultrasound. One of the reasons why screening has not been shown to be effective is that the serological AFP test currently used has limited sensitivity.[19,20] Thus, we also need better serological tests to improve screening protocols.

4. Molecular Markers

The only serological marker widely used for the screening and diagnosis of HCC is AFP. This protein is synthesized in large quantities during embryonic development by the yolk sac and liver.[21,22] AFP concentration in the serum decreases gradually after birth to <10 ng/mL by the time a child is 12–18 months old; in females, it reappears in maternal serum during pregnancy. Increased circulating AFP has been associated with HCC, gastric carcinoma, lung cancer, pancreatic cancer, biliary tract cancer, and testicular carcinoma.[21]

A recent systematic review of the published literature shows that at a cut-off value of 20 ng/mL AFP displays between 41–65% sensitivity and 80–94% specificity (HCC vs CLD) for the diagnosis of HCC;[23] for small tumors, sensitivity is around 40%.[22,24] AFP can also be moderately elevated in some patients with benign liver disease.[25] If a higher cut-off value is used, the specificity of the test will increase but, obviously, the sensitivity will be reduced.[23] Due to the limited sensitivity, particularly for small tumors, the usefulness of AFP measurement as a diagnostic test and surveillance tool for patients at risk for HCC has been questioned.[26,27] It has been proposed that the only circumstance in which the measurement of AFP is justified is for the confirmation of an initial diagnosis based on an imaging technique.[27]

Given the limitations of the AFP test, the search for more sensitive serological markers for HCC has continued. Other molecules have been investigated as potential markers, including des-gamma carboxy-prothrombin (DGCP, also called protein induced

by vitamin K absence or antagonist-II [PIVKA-II]),[28-31] and the AFP variant AFP-L3.[32] None of these markers have been conclusively shown to be more sensitive than AFP.[33] DGCP is currently being used in Japan, but has not been recommended by the European Association for Study of the Liver (EASL).[34]

HCCs are very heterogeneous tumors,[35] therefore the difficulties in finding good tumor markers are not surprising. In fact, as the evidence for HCC heterogeneity accumulates, it is increasingly evident that it is unlikely that a single marker will be found to display both very high sensitivity and specificity. Thus, it is reasonable to propose that an optimal serological test for HCC will be based on the simultaneous measurement of two or three highly specific serological markers.

5. Glypican-3, a Novel Serological and Histochemical Marker for Hepatocellular Carcinoma

The glypicans are a family of heparan sulfate proteoglycans (HSPGs) linked to the exocytoplasmic surface of the plasma membrane by a glycosyl-phosphatidylinositol anchor.[36-39] To date, six glypicans have been identified in mammals (GPC1 to GPC6).[39,40] Genetic and functional studies performed in *Drosophila, Xenopus, Zebrafish*, and mammals have demonstrated that glypicans can increase the signaling activity of Wnts, hedgehogs, and bone morphogenetic proteins (BMPs).[41-52] In the specific case of Wnts, glypicans have been reported to stimulate both the canonical and non-canonical pathways.[42,47,48] Since Wnts are known to bind to heparan sulfate,[53] it has been proposed that the stimulatory activity of glypicans is based on their ability to act as facilitators of the interaction between Wnts and their receptors.[42] This hypothesis has been supported by the finding that several Wnts can co-immunoprecipitate with glypicans.[48,49,54] In addition to acting as facilitators of ligand-receptor interactions, glypicans may play a role during development in the transport of Wnts, hedgehogs, and BMPs for the purpose of morphogen gradient formation.[52,55]

In 1997 Hsu et al.[56] reported the identification of a transcript that was up-regulated in HCC. This transcript, which had been previously named MXR7,[57] was later called glypican-3 (GPC3).[58] These investigators found that GPC3 messenger RNA (mRNA) was expressed in 143 of 191 (75%) primary and recurrent HCCs, but in only 5 of 154 (3%) normal livers. In addition, no GPC3 expression was detected in three hepatocellular adenomas or in six cholangiosarcomas.[56]

Three other reports confirming the over-expression of GPC3 mRNA in HCC have been published more recently.[59-61] In one of these studies the authors compared the levels of GPC3 mRNA in HCC versus normal liver, focal nodular hyperplasia (a benign lesion), and liver cirrhosis.[59] They found that GPC3 mRNA values in focal nodular hyperplasia and liver cirrhosis were similar to those of normal liver, and that levels of GPC3 in HCCs were above the mean value of the non-malignant groups in 25 of 30 (83%) patients. Based on these results the investigators proposed that GPC3 could be a useful marker to differentiate between benign and malignant liver tissue.[59]

In our laboratory we decided to investigate whether the increase in GPC3 mRNA found in HCC by other investigators was accompanied by an equivalent increase in the levels of the corresponding protein. To this end, we generated monoclonal antibodies against the C-terminus of GPC3 that are able to stain paraffin-embedded tissue sections. Using these antibodies we found that GPC3 was expressed in 21 of 29 (72%) of HCCs, whereas it was not detectable in hepatocytes from normal liver and benign liver diseases[62] (figure 1). These results are clearly consistent with those from the mRNA studies discussed above. Another interesting aspect of the immunohistochemical study was the finding that GPC3 is expressed in small tumors (<3cm), a property that is very important for a tumor marker to be used for diagnostic purposes. Overexpression of GPC3 in HCC tissue sections at the protein level has been also reported by another laboratory.[63]

Since the immunostaining pattern of GPC3 in HCC tissue sections showed that this glypican can also be seen in the intercellular space, we decided to investigate whether GPC3 could be found in the sera of HCC patients. To this end, we established a sandwich ELISA, using one of our monoclonal antibodies (1G12) and an anti-GPC3 polyclonal antibody.[62] In our initial study, we found that whereas GPC3 was undetectable in the serum of 53 healthy donors and 18 patients with hepatitis, this glypican could be found in the serum of 18 of 34 (53%) of the patients with HCC. In addition, only 1 of 20 patients with hepatitis plus liver cirrhosis displayed elevated levels of GPC3.[62] Thus, the specificity of the serological GPC3 test for the diagnosis of HCC in a population of patients with CLD was very high in this study (97%). On the other hand, sensitivity was limited. In this regard it is important to note that we also measured serological AFP in the same set of HCC patients and found that, at a cut-off level of 20 ng/mL, AFP was elevated in 59% of samples. Interestingly, in most cases elevated GPC3 values did not correlate with elevated AFP values. As a consequence, at the standard cut-off value of 20 ng/mL for AFP, the serological level of at least one of the two markers was

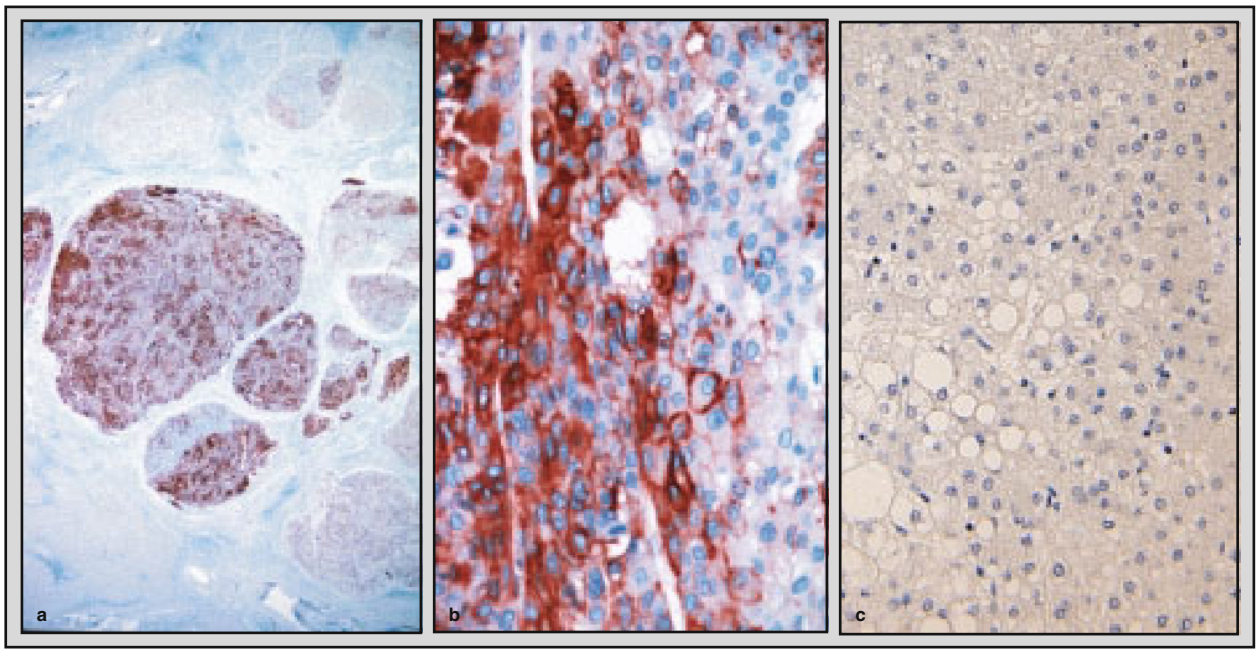

Fig. 1. Immunohistochemical staining of hepatocellular carcinoma sections with the anti-glypican-3 (GPC3) 1G12 monoclonal antibody. (**a**) Low power picture of a GPC3-positive multinodular hepatocellular carcinoma surrounded by GPC3-negative non-malignant areas, including hepatocytes, blood vessels, and bile ducts; (**b**) high power picture of the same stained hepatocellular carcinoma section, showing membrane and cytoplasmic staining; and (**c**) GPC3-negative dysplastic nodule.

elevated in 82% of HCC patients. These results suggest that the sensitivity of the diagnostic test can be significantly improved without compromising specificity by the simultaneous measurement of both GPC3 and AFP.

Two other recent studies have confirmed our results with regard to the potential role of GPC3 as a marker for HCC.[64,65] In addition, both reports confirmed the lack of overlap between GPC3 and AFP values in HCC patients. In the Nakatsura et al.[64] study, the sensitivity of the GPC3 test was somewhat lower than in our study (40%). However, it has to be noted that Nakatsura et al.[64] used for their GPC3 ELISA a commercially available polyclonal antibody which is clearly not very specific, since it detects multiple additional protein bands in Western blots that do not correspond to GPC3 (see figure 2 in Nakatsura et al.[64]). Moreover, to standardize their assay they used supernatants of GPC3-expressing cells, which contain many other proteins in addition to GPC3.[64] This could increase the background in the ELISA and reduced the sensitivity of the assay.

In the study by Hippo et al.,[65] the sensitivity of the GPC3 test was similar to that of our study. Surprisingly, however, the authors claimed that they could not detect GPC3 in the serum of HCC patients if they used a monoclonal antibody raised against the C-terminus. They proposed, therefore, that in HCC cells GPC3 is released by a protease that leaves the C-terminus of GPC3 bound to the cells. Our results, however, indicate that at least a large proportion of serum GPC3 contains the C-terminus, since by using an antibody against such terminus we could detect GPC3 in 53% of patients by using ELISA. In addition, we can also detect GPC3 in serum by Western blot analysis using the same antibody.[66]

6. Conclusion

Data reported by several laboratories indicate that GPC3 is specifically overexpressed in a majority of patients with HCCs, and that GPC3 could be used as a histological and serological marker for HCC. Furthermore, results published to date suggest that, although the sensitivity of the serological GPC3 test is limited, the combined used of AFP and GPC3 could provide a highly specific and sensitive diagnostic test for HCC. It is also possible that the combined use of GPC3 and AFP would provide an improved test to screen the large number of cirrhotic patients that are at risk of developing HCC. Certainly, further studies are required to test this hypothesis.

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