Glypican-3 level assessed by the enzyme-linked immunosorbsorbent assay is inferior to alpha-fetoprotein level for hepatocellular carcinoma diagnosis

Yejoon Jeon, Eun Sun Jang*, Yun Suk Choi, Jin-Wook Kim, and Sook-Hyang Jeong

Department of Internal Medicine, Seoul National University College of Medicine, Seoul National University Bundang Hospital, Seongnam, Korea

Background/Aims: Glypican-3 (GPC3) protein is highly expressed in hepatocellular carcinoma (HCC) tissue. It has been suggested as a diagnostic biomarker, but its inconsistent performance means that it requires further assessment. We therefore investigated the diagnostic value of the plasma GPC3 level compared to the alpha-fetoprotein (AFP) level as a diagnostic biomarker of HCC.

Methods: We enrolled 157 consecutive patients with newly diagnosed HCC and 156 patients with liver cirrhosis (LC) as the control group. GPC3 plasma levels were measured using two commercially available enzyme-linked immunosorbent assays (ELISAs, named as Assay 1 and 2), and AFP levels were measured using an enzyme-linked chemiluminescent immunoassay. The diagnostic accuracy was analyzed using the receiver operating characteristics (ROC) curve.

Results: Plasma GPC3 levels in HCC patients were very low (0–3.09 ng/mL) in Assay 1, while only 3 of the 157 patients (1.9%) showed detectable GPC3 levels in Assay 2. The median GPC3 level was not significantly elevated in the HCC group (0.80 ng/mL) compared with the LC group (0.60 ng/mL). The area under the ROC curve (AUC) for GPC3 was 0.559 in Assay 1. In contrast, the median AFP level was significantly higher in HCC (27.72 ng/mL) than in LC (4.74 ng/mL), with an AUC of 0.729.

Conclusions: The plasma level of GPC3 is a poor diagnostic marker for HCC, being far inferior to AFP. The development of a consistent detection system for the blood level of GPC3 is warranted. (Clin Mol Hepatol 2016;22:359-365)

Keywords: Alpha-fetoprotein; Biomarkers; Diagnosis; Glypican-3; Hepatocellular carcinoma; Sensitivity, Specificity

INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for 80-90% of liver cancer, which is the 6th most frequent cancer and the third leading cause of all cancer related deaths.1,2 While typical diagnostic methods for HCC include imaging methods and histological analysis, in the former it is difficult to detect and differentiate small nodules, whereas the latter is invasive and holds high-risk, especially in patients with cirrhotic backgrounds. Blood alpha-fetoprotein (AFP) levels have been used as a marker for HCC diagnosis and to supplement radiological findings. The Asian Pacific Association for the Study of the Liver guidelines...
recommend AFP as a tumor marker for HCC with a cutoff value at 200 ng/mL in addition to radiological examinations. However, the current guidelines of the American Association for the Study of Liver Disease and the European Association for the Study of the Liver have excluded AFP based on its limited accuracy. AFP has moderate sensitivity for HCC, but lacks specificity as it is elevated in benign hepatic condition including cirrhosis and hepatitis. Therefore, additional biomarkers that are HCC-specific and -sensitive require investigation.

The heparin sulfate proteoglycan glypican-3 (GPC3) is a member of the glypican family and is bound to the membrane through a glycosphatidylinositol (GPI) anchor. It is highly expressed in HCC cells and promotes HCC proliferation through the canonical Wnt pathway, and therapeutic methods that target GPC3 are currently being investigated. However, GPC3 elevation in the sera of HCC patients is less conclusive. Initial studies reported high sensitivity of GPC3 as a specific biomarker for HCC. Nonetheless, subsequent studies have led to inconsistent results, questioning plasma GPC3’s value as a diagnostic biomarker. The aim of this study was to address this concern by assessing and comparing the diagnostic accuracy of GPC3 and AFP plasma level in a South Korean HCC population.

**PATIENTS AND METHODS**

**Patients**

The study design was a cross sectional, retrospective, case-control study including 157 HCC patients and a control group of 156 liver cirrhosis (LC) patients at the Seoul National University Bundang Hospital (Seongnam, Republic of Korea) from August 2006 through September 2013. This study was approved by the Seoul National University Bundang Hospital’s Institutional Review Board (IRB, B-1307/210-006). Informed consent was confirmed by the IRB and received from all HCC patients. LC plasma samples were derived from repository samples, and thus informed consent was waived by the IRB.

HCC was diagnosed by histological and imaging findings outlined by American Association for the Study of Liver Disease guidelines and staged by the Barcelona Clinic Liver Cancer (BCLC) system. Patients with double primary cancer in non-liver organs or those who underwent intent-to-cure treatment (resection, liver transplantation, radiofrequency ablation, transarterial chemoembolization, or percutaneous ethanol injection) were excluded from this study. LC was diagnosed by histological examination or by one or more clinical findings of portal hypertension: 1) cirrhotic appearance of the liver with splenomegaly on imaging studies (ultrasonography, computed tomography or magnetic resonance image), 2) thrombocytopenia (platelet < 120,000/mm³), 3) presence of esophagogastric varices on endoscopy, 4) presence of ascites, and 5) presence of portosystemic encephalopathy. LC patients for whom the presence of HCC was not evaluated within six months before enrollment were excluded.

**Sample storage and measurement of AFP and GPC3**

Blood samples were collected from HCC patients before curative treatment and from LC patients at the time of clinic visit. Plasma aliquots were stored at -70°C until time of measurement. Plasma AFP was measured using an automated enzyme-linked chemiluminescent immunoassay (ELICA). Plasma GPC3 levels

| Category              | Assay 1                  | Assay 2                  |
|-----------------------|--------------------------|--------------------------|
| Manufacturer          | USCN Life Science Inc.   | R&D Systems, Inc.        |
| Capture antibody      | Monoclonal mouse         | Monoclonal mouse         |
| Detection antibody    | Polyclonal rabbit        | Polyclonal sheep         |
| Plate                 | Pre-coated with capture antibody | Manual capture antibody coating |
| Incubation temperature| 37°C                     | Room temperature         |
| Biotin binding protein| Avidin                   | Streptavidin             |
| Wash                  | Aspirate but no wash between adding sample and detecting antibody | Aspirate and wash between adding sample and detecting antibody |
| Detection range       | 0.156 ng/mL – 10 ng/mL   | 0.312 ng/mL – 20 ng/mL   |
| Sample dilution factor| 1:1                      | 1:100                    |

ELISA, enzyme-linked immunosorbent assay.
were assessed using two different commercially available enzyme-linked immunosorbent assay (ELISA) kits named as Assay 1 and 2 (Assay 1: USCN Life Science Inc., Wuhan, China and Assay 2: R&D Systems, Inc., Minneapolis, MN, USA), which were compared in Table 1. Tests were performed in accordance to manufacturer’s instructions and in duplicates by two experienced researchers (YSC and YJ). In Assay 2, a sample dilution of 1:1 did not yield results within the range studied. Sample dilutions (1:1, 1:10, 1:50, 1:100, 1:500, and 1:1000) showed that 1:100 yielded consistent and peak GPC3 values. This dilution factor was therefore used for all samples.

**Statistical analysis**

Pearson’s \( \chi^2 \) (categorical variables), Student’s independent t-test (continuous parametric variables), and Mann-Whitney U test (continuous nonparametric variables) were used to compare HCC and LC groups. Significance was defined as \( P<0.05 \). Receiver operating characteristic (ROC) curve and area under the curve (AUC) analyses were performed to compare diagnostic accuracies of

| Table 2. Clinical characteristics of the study population |
|---------------------------------------------|
| **Total** | **LC** | **HCC** | **P-value** |
| **Age, year** | 58.8 (11.5) | 56.7 (10.8) | 60.8 (11.8) | 0.002 |
| **Gender** | 217 (69.3) | 90 (57.7) | 127 (80.9) | <0.001 |
| **BMI, kg/m** | 23.88 (4.12) | 23.92 (4.6) | 23.84 (3.6) | 0.860 |
| **Etiology** | 20 (6.4) | 3 (1.9) | 17 (10.8) | |
| **HBV** | 222 (70.9) | 116 (74.4) | 106 (67.5) | |
| **HCV** | 54 (17.3) | 37 (23.7) | 17 (10.8) | |
| **Cryptogenic** | 14 (4.5) | 0 (0.0) | 14 (8.9) | |
| **Others** | 3 (0.9) | 0 (0.0) | 3 (1.9) | |
| **Child-Pugh class** | 261 (83.0) | 133 (85.3) | 128 (81.5) | 0.516 |
| **A** | 45 (14.8) | 19 (12.2) | 26 (16.6) | |
| **C** | 7 (2.3) | 4 (2.5) | 3 (1.9) | |
| **Underlying liver disease** | 10 (3.2) | 0 (0.0) | 10 (6.4) | <0.001 |
| **Liver cirrhosis** | 293 (93.6) | 156 (100.0) | 137 (87.3) | |
| **Tumor characteristics** | 21 (13.4) | 56 (35.7) | 11 (7.0) | |
| **BCLC stage** | 62 (39.5) | 7 (4.5) | 18 (11.5) | 32 (20.4) |
| **Diffuse type** | 18 (11.5) | 32 (20.4) |
| **With major PVI** | 14 (8.9) | 3 (1.9) |
| **LC, liver cirrhosis; HCC, hepatocellular carcinoma; BMI, body mass index; HBV, hepatitis B virus; HCV, hepatitis C virus; BCLC, barcelona clinic liver cancer; PVI, portal vein invasion.**
| *Mean (Standard Deviation). |
| †Number (Percentage). |
GPC3 and AFP. The maximum sum of sensitivity and specificity on the ROC curve determined the GPC3 cut-off level. All statistical analyses were performed via SPSS Statistics 17.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Characteristics of HCC patients and control group of cirrhosis patients

The clinical characteristics of the study population (157 HCC and 156 LC) were summarized in Table 2. The HCC group had a higher proportion of males and was older than the LC control group. In both groups, hepatitis B virus (HBV) was the most common etiology (67.5% in HCC and 74.4% in LC). Among the HCC patients, 6.4% and 87.3% had underlying liver diseases of chronic hepatitis and liver cirrhosis, respectively. There were no significant differences in Child-Pugh class between the two groups. BCLC staging was 0 in 13.4%, A in 35.7%, B in 7.0%, C in 39.5% and D in 4.5% of the HCC patients. Major portal vein invasion was observed in 20.4% of the HCC patients.

Plasma levels of GPC3 and AFP in HCC and LC patients

The median and mean levels of GPC3 and AFP were summarized in Table 3.

In Assay 1, the median GPC3 value for HCC patients was 0.80 ng/mL (range, 0.00 ng/mL to 3.09 ng/mL) for HCC patients, and for LC patients was 0.60 ng/mL (range, 0.07 ng/mL to 7.40 ng/mL). These differences were not significant (P=0.255).

In Assay 2, all but 3 HCC samples were "Out of Range". None of the LC samples reached concentrations within the range of the assay. Because of the lack of HCC patients with detectable GPC3 levels, AUC analysis was not performed based on this data. Clinical characteristics and GPC3 concentrations based on Assay 1 and 2 of these samples were listed in Table 4.

Median plasma AFP level was 27.72 ng/mL (range, 0.72 ng/mL to 42,000.0 ng/mL) in HCC patients and 4.74 ng/mL in LC patients (range, 1.08 ng/mL to 145.08 ng/mL), which showed significantly elevated levels in HCC patients compared to LC patients (P<0.001).

In addition, when comparing LC GPC3 and AFP median values to just that of early HCC patients (BCLC 0–B, n=88), again only AFP was significantly elevated in the latter (GPC3 0.65 ng/mL P=0.916, AFP=11.94 ng/mL P<0.001; data not shown).

| Table 3. Plasma GPC3 and AFP levels in HCC and LC patients |
|------------------------------------------------------------|
|               | Total (N=313) | LC (N=156) | HCC (N=157) | P-value |
|----------------|---------------|------------|-------------|---------|
| AFP level      |               |            |             |         |
| Median         | 7.44          | 4.74       | 27.72       | <0.001  |
| Range          | 0.72-42000    | 1.08-145.08| 0.72-42000.00 |
| Mean           | 1960.79       | 9.90       | 3899.26     |         |
| SD             | 8026.26       | 17.72      | 11011.53    |         |
| GPC3 in Assay 1|               |            |             | 0.255   |
| Median         | 0.65          | 0.60       | 0.80        |         |
| Range          | 0-7.40        | 0.07-7.40  | 0-3.09      |         |
| Mean           | 0.89          | 0.86       | 0.92        |         |
| SD             | 0.78          | 0.86       | 0.69        |         |
| GPC3 in Assay 2*|              |            |             |         |
| Median         | 14.79         | All Results| 14.79       |         |
| Range          | 10.59-83.16   | Out of     | 10.59-83.16 |         |
| Mean           | 36.18         | Range      | 36.18       |         |
| SD             | 40.74         |            | 40.74       |         |

All values are in ng/mL.

GPC3, Glypican-3; AFP, Alpha-fetoprotein; HCC, Hepatocellular carcinoma; LC, Liver cirrhosis; SD, Standard deviation.

*Only three HCC samples had detectable GPC3 values.
Diagnostic Accuracy of GPC3 and AFP

Using the ROC curves (Fig. 1), AUCs were calculated to assess the diagnostic accuracy of plasma AFP and GPC3 for HCC. Based on maximum sum of sensitivity and specificity, a GPC3 cut-off of 0.61 ng/mL was determined. At this cut-off, GPC3 had a sensitivity of 60% and a specificity of 52%. At a cut-off of 20 ng/mL, AFP had a sensitivity of a 55% and specificity 91%. The AUC was 0.559 and 0.729 for GPC3 and AFP respectively, indicating AFP as a stronger diagnostic tool. Combining the two markers only provided a marginal change; GPC3 and AFP (at cut-offs of 0.61 ng/mL and 20 ng/mL respectively) had an AUC of 0.744, sensitivity of 55%, and specificity of 91%. Furthermore, when comparing the LC group to the early HCC subgroup, at the same cut-offs, AFP (AUC=0.665) was a superior diagnostic biomarker compared to GPC3 (AUC= 0.527).

DISCUSSION

We set out to determine whether GPC3 could be useful as a diagnostic biomarker for HCC in comparison to the traditional AFP in an HBV endemic, South Korean background. While AFP levels were significantly elevated in HCC patients compared to LC patients, plasma GPC3 levels were either very low or undetectable. Our study reported comparable sensitivity, but much lower specificity and AUC values for GPC3 (52% and 0.559) compared to other studies (84% and 0.762).

The ROC determined cut-off value for GPC3 of 0.61 ng/mL was lower than most reported values, though it has been reported to range from 3.9 pg/mL to 300 ng/mL.

Meanwhile, AFP sensitivity, specificity, and AUC were comparable to reported results.

Overall, the plasma level of GPC3 was a poor diagnostic biomarker for HCC compared to AFP.

Though we used 2 commercially available GPC3 assays, GPC3 values detected by the two assays showed discrepancies. Assay 1 resulted in lower GPC3 values in HCC patients than those reported by previous studies, although some studies have reported similarly low values. In Assay 2, only 1.9% of the HCC patients had detectable values in this study. The technical differences between the two assays included sample dilution factor and washing method. These differences may have contributed to discrepancies between the two assays.

Since first reported to be present in the plasma of 53% HCC patients, GPC3 by ELISA is inferior to AFP for HCC diagnosis.
patients but undetectable in healthy liver controls, GPC3 has been studied as a biomarker for HCC to replace or complement AFP. However, subsequent studies have yielded inconsistent results as summarized in Table 5, showing inter- and intra-ELISA variances. Differences in capture and detection antibodies could explain inter-method differences. While Capurro et al. developed antibodies targeting the COOH-terminus for their ELISA, Hippo et al. used antibodies targeting the NH2-terminus. Though Capurro et al. and Hippo et al. reported similar elevated rates of GPC3 in HCC patients (53% and 51%, respectively), their scales were completely different (0-2924 ng/mL vs 0-~60 ng/mL). The nature of plasma GPC3 may impact ELISA utility. Plasma GPC3 can be produced when an extracellular lipase (Notum) cleaves the GPI anchor of membrane bound GPC3 or when secreted, possibly by endogenous Notum or GPI-phospholipase D, by the cell. Various species of GPC3 may also be found, as Hippo et al. was able to detect a 50-kDa fragment produced by a cleavage site in the COOH-terminal in addition to the commonly detected glycanated GPC3. Furthermore, plasma GPC3 competitively binds with several growth factors, which could interfere with antibody binding. Given these possibilities, it is clear that a uniform understanding of plasma GPC3 has not been reached. There may be ways to circumvent these proposed obstacles. Comparison of cell lysate and supernatant could help differentiate membrane bound and secreted GPC3; an antibody that targets the NH2 terminal may detect both the 50kDa and glycanated forms of GPC3; and a dilution of the plasma could help prevent interference of antibody binding. Nonetheless, for plasma GPC3 to be a universal biomarker, the biochemistry behind plasma GPC3 concerning its origin as well as structure(s) must be elucidated. Finally, heterogeneity in HCC composition, ethnicity, etiology, and clinical manifestation may lead to variability. Moreover, meta-studies point out methodological issues such as differences in inclusion and exclusion criteria or use of reference standard tests.

The limitations of this study were that it was a single center, cross-sectional design without external validation samples, no assessment of concurrent GPC3 tissue expression, and usage of plasma samples. It also included only 2 commercially available ELISA.

In conclusion, plasma GPC3 level measured by currently available 2 ELISA kits clearly failed to be a useful diagnostic biomarker for HCC compared to AFP. Persistent inconsistency in GPC3 values questions its utility. Future studies regarding the development of more reliable detection systems perhaps including blood GPC3 levels are warranted.

Table 5. Summary of reported studies measuring blood GPC3 levels in HCC patients using ELISA

| Author (year) | Country | HCC cases | GPC3 mean | GPC3 median | GPC3 range | ELISA kit |
|---------------|---------|-----------|-----------|-------------|------------|-----------|
| Beale et al. (2008) | UK | 50 | 161.41 ng/mL | 56.57 ng/mL | BioMosaics limited (Burlington, VT, USA) |
| Yasuda et al. (2010) | Japan | 200 | 924.8 pg/mL | | |
| Lee et al. (2014) | South Korea | 120 | 75.8 ng/mL | 21.7-482.5 ng/mL | Cusabio (Wuhan, China) |
| Wang et al (2013) | China | 84 | 4.55 ng/mL | | |
| Badr et al. (2014) | Egypt | 30 | 551.47 µg/mL | | USCN Life Science Inc. (Wuhan, China) |
| El Gawad et al. (2014) | Egypt | 40 | 7.7 ng/mL | | |
| El-Shenawy et al. (2012) | Egypt | 85 | 1,646.3 ng/mL | | |
| Ozkan et al. (2011) | Turkey | 75 | 3.9 pg/mL | | EIAab Science Co. (Wuhan China) |
| Capurro et al. (2003) | Canada | 34 | 0-2924 ng/mL | | Non-Commercial |
| Chen et al. (2013) | China | 155 | 99.94 ng/mL | 15.11 ng/mL | 0–2400.00 ng/mL |
| Hippo et al. (2004) | Japan | 69 | 4.84 ng/mL | | |

*Reported to use same procedure as (5).* GPC3, glypican-3; HCC, hepatocellular carcinoma; ELISA, enzyme-linked immunosorbent assay.
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Conflicts of Interest
The authors have no conflicts to disclose.

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