A comparative evaluation of Golgi protein-73, fucosylated hemopexin, α-fetoprotein, and PIVKA-II in the serum of patients with chronic hepatitis, cirrhosis, and hepatocellular carcinoma

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Abstract

Background: Golgi protein-73 (GP73) and fucosylated proteins have been proposed as potential serum markers for liver disease and/or hepatocellular carcinoma (HCC). The purpose of this study was to compare the sensitivity and specificity of serum GP73 and fucosylated hemopexin (Fuc-HPX) with α-fetoprotein (AFP) and with protein induced by the absence of vitamin K or antagonist-II (PIVKA-II) for diagnosing chronic hepatitis, cirrhosis, and HCC.

Methods: The concentration of GP73 in human sera was determined using an enzyme-linked immunosorbent assay employing mouse monoclonal and rabbit polyclonal GP73 antibodies. Fuc-HPX was detected using a lectin chemiluminescence-linked immunosorbent assay using a mouse monoclonal anti-hemopexin antibody and Aleuria aurantia lectin. A total of 229 serum samples from patients with chronic hepatitis, cirrhosis, and HCC, as well as from normal individuals were evaluated using these four markers.

Results: GP73 and Fuc-HPX showed significantly higher values in samples from patients with cirrhosis and HCC than in samples from patients with hepatitis and from normal individuals. The areas under the curves (AUCs) for GP73, Fuc-HPX, AFP, and PIVKA-II were 0.90, 0.77, 0.74, and 0.88, respectively, for liver cirrhosis and HCC samples vs. hepatitis and normal samples. The AUCs of GP73, Fuc-HPX, AFP, and PIVKA-II were 0.78, 0.72, 0.81, and 0.90, respectively, for HCC samples vs. all other samples.

Conclusions: PIVKA-II showed superior sensitivity and specificity for HCC compared with the other three markers.

GP73 may be useful for detecting cirrhosis as a risk factor for HCC. Fuc-HPX showed inferior sensitivity and specificity compared to the other markers.

Keywords: cirrhosis; fucosylation; Golgi protein-73; hepatocellular carcinoma; liver disease.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in men and the eighth most common cancer in women worldwide (1, 2). It is highly prevalent in the Asia-Pacific region and in Africa, and is increasing in Western countries (3). HCC is frequently detected at an advanced stage, having a poor diagnosis, and often arises in a background of chronic liver disease and cirrhosis (4). Chronic infections with hepatitis B virus (HBV) and hepatitis C virus (HCV) are risk factors for HCC. HCV infection is associated with the highest incidence of HCC in patients with cirrhosis. The risk for the 5 year cumulative incidence of HCC is higher by a factor of approximately two in patients with HCV-related cirrhosis in Japan, compared with patients with HBV-related cirrhosis in Taiwan and Singapore (4). In such patients with hepatitis, surveillance strategies for screening for early HCC are necessary since screening facilitates the early identification of tumors.

Several serum based tumor markers for HCC have been developed commercially, or are under investigation. α-fetoprotein (AFP) is widely used in clinical practice worldwide for screening patients at high-risk for HCC, for the diagnosis and monitoring of HCC patients in conjunction with ultrasound to detect early recurrence, for monitoring the response to therapy, and for detecting early relapse. However, AFP can be produced under many circumstances, including other liver diseases, and is not present in all patients with HCC. Protein induced by the absence of vitamin K or antagonist-II (PIVKA-II), also known as des-γ-carboxy prothrombin (DCP), is widely used with AFP in Japan during and after HCC treatment to predict adverse outcomes, and to detect early recurrence and potential malignancy (5–7). Falsely increased PIVKA-II concentrations are found in patients with severe obstructive jaundice due to intrahepatic cholestasis, or under conditions in which the action of vitamin K is impaired in individuals with long standing vitamin K deficiency, and in those who have ingested Warfarin or wide spectrum antibiotics (8).
Recently, resident Golgi protein-73 (GP73) has been proposed as a candidate serum marker for the diagnosis of HCC (9–11). GP73 is widely expressed in normal epithelial cells from several tissues. Its expression is upregulated in hepatocytes from patients with viral and non-viral liver disease. GP73 regulation and the biological significance of increased GP73 in sera are unclear. Upregulated intracellular GP73 expression enhances its intracellular trafficking through the endosomal pathway, which provides the opportunity for endoproteolytic cleavage of GP73, resulting in the secretion of truncated GP73. The potential utility of GP73 as a serum marker for the diagnosis of liver disease has also been reported (12).

The Lens culinaris hemagglutinin reactive fraction of AFP (AFP-L3), a fucosylated glycoform of AFP, is used in Japan as a marker to represent the degree of biological malignancy of HCC (13, 14). An AFP-L3 assay has also been approved by the US Food and Drug Administration for diagnosis of HCC because of its higher specificity than AFP. An association of changes in glycosylation with the development of cirrhosis and HCC has been reported (15). The altered glycosylation of serum glycoproteins, such as fucosylated kininogen and fucosylated α1-antitrypsin, may act as potential biomarkers when used independently or in combination with other HCC markers (16, 17). The level of fucosylated hemopexin (Fuc-HPX) in the Lens culinaris hemagglutinin lectin bound serum fraction has been reported to show good diagnostic accuracy for HCC in a study using a small sample set, and to be a new potential biomarker for HCC (18). Hemopexin (HPX) is expressed primarily in the liver, and is also an abundant and acute phase protein in human serum (19). This protein has five glucosamine oligosaccharides that are N-linked to the acceptor sequence, Asn-X-Ser/Thr (20), and two core fucosylation sites of HPX in human serum have been identified using a proteomics approach (21). Aleuria aurantia lectin (AAL) is a fungal protein composed of two identical 312-amino acid subunits that specifically recognizes fucosylated glycans, and is widely used as a specific probe for fucose (22).

In this study, we developed an enzyme-linked immunosorbent assay (ELISA) to determine the concentration of GP73 in serum quantitatively, and a chemiluminescence-linked immunosorbent assay (CLISA) to detect Fuc-HPX using AAL. We evaluated the sensitivity and specificity of these two potential new markers for diagnosing chronic hepatitis, cirrhosis, and HCC compared to that of AFP and PIVKA-II.

Materials and methods

Participants

Patient serum and clinical information between August 1994 and July 2009 were utilized with the approval of the Johns Hopkins University Institutional Review Board and commercial sample vendors. A total of 229 serum samples from 70 patients with HCC, 35 patients with liver cirrhosis, 52 chronic hepatitis patients, including those infected with HBV (n = 13), those infected with HCV (n = 35), those with autoimmune hepatitis (n = 3), and those with steatohepatitis (n = 1), along with 12 healthy individuals (normal) from Johns Hopkins University were examined. All the HCC samples were from patients who were not being treated for HCC. The ethnic compositions (Asian, Black, Hispanic, White, and unknown) of the HCC, cirrhosis, and chronic hepatitis groups were 10:10:2:48:0, 3:8:0:23:1, and 4:23:0:25:0, respectively. The majority of three sample groups were White, a major ethnic group in the general US population. In addition, 60 commercial serum samples from normal blood donors from ProMedDx (Norton, MA, USA) were examined using GP73, Fuc-HPX, AFP, and PIVKA-II assays.

A total of 130 samples from 39 patients with HCC, 20 patients with liver disease, such as autoimmune hepatitis (n = 3), non-alcoholic steatohepatitis (NASH; n = 7), alcoholic hepatitis (n = 1), cirrhosis (n = 3), and unknown liver disease (n = 6), along with 71 normal blood donors from ProMedDx, Bioreclamation (Hicksville, NY, USA), Clinical Research Center of Cape Cod (Hyannis, MA, USA), and QC Products (Pompano Beach, FL, USA) were analyzed using Fuc-HPX and total HPX assays.

GP73 ELISA

ELISA plates (Maxisorp, Thermo Fisher, Waltham, MA, USA) were coated with mouse anti-GP73 monoclonal antibody, 14H4-23 (Drexel University, Doylestown, PA, USA) and blocked with a PBS solution containing 1% bovine serum albumin (BSA). Diluted serum samples (1:100) were added to the wells, incubated for 1 h at 37°C, and then each well was washed five times with 350 μL of 10 mM Tris buffer containing 0.15 M NaCl and 0.01% Tween-20 (TNT). Rabbit anti-GP73 polyclonal antibody (Drexel University) was added to the wells, which were then incubated for 1 h at 25°C, and then washed. Goat anti-rabbit IgG (H + L) HRPO conjugate (Southern Biotech, Birmingham, AL, USA) was added, and the samples were incubated for 30 min at 25°C, and then washed. A volume of 100 μL of OPD solution was added, and the samples were then incubated for 30 min at 25°C. After adding a 1 M H2SO4 solution, the OD492 and OD630 values were read using a microplate reader (Thermo Labsystems, Vantaa, Finland). The concentrations were determined using a calibration curve of purified recombinant GP73 in HEK293 (Abbott Laboratories, Abbott Park, IL, USA) in a PBS solution containing 1% BSA. The final GP73 values in the sera were reported by multiplying by a dilution factor of 100. This enzyme immunoassay uses two anti-GP73 antibodies and can possibly be applied to an automated immunoassay system similar to other comparative markers, such as AFP and PIVKA-II.

Fuc-HPX CLISA

Mouse anti-HPX antibody, ABS 013-04 (Bioporto Diagnostics, Gentofte, Denmark) was treated with a 10 mM periodate solution for 1 h at 37°C, and the oxidized antibody was coated onto ELISA plates (Maxisorp, Thermo Fisher) and incubated at 4°C overnight. Diluted serum samples (1:250) were added to the wells, which were incubated for 1 h at 37°C, and each well was washed five times with 350 μL of 10 mM TNT. Biotinylated AAL (Vector Laboratories, Burlingame, CA, USA) was incubated for 1 h at 25°C and then washed. Acridinium ester (Abbott Laboratories) conjugated streptavidin was incubated for 30 min at 25°C and then washed. The trigger and pretrigger solutions (Abbott Laboratories) were dispensed, and a signal was detected using a Luminoskan Ascent luminometer (Thermo Fisher). The signals from all the samples were compared with signals detected from commercial normal human serum (Sigma-Aldrich, St. Louis, MO, USA), and reported using the signal-to-noise ratio (S/N).
**Total HPX ELISA**

Mouse anti-HPX antibody, ABS 013-04 (Bioporto Diagnostics) in carbonate buffer was coated onto an ELISA plate (Maxisorp, Thermo Fisher), kept overnight at 4°C, and blocked with a PBS containing 3% BSA. Diluted serum samples (1:20,000) were added to the wells and incubated for 1 h at 37°C. Each sample was washed five times with 350 μL of 10 mM TNT. Rabbit anti-HPX polyclonal antibody (Assaypro, St. Charles, MO, USA) was then added and the wells were incubated for 1 h at 25°C and then washed. Goat anti-rabbit IgG (H + L) HRPO conjugate (Southern Biotech) was added, and the sample was incubated for 30 min at 25°C. A volume of 100 μL of OPD solution was added, and the samples were incubated for 30 min at 25°C. After adding a 1 M H2SO4 solution, the OD492 and OD650 values were read using a microplate reader (Thermo Labsystems). The results were calculated from a calibration curve using purified HPX (Assaypro) in 10 mM TNT. The final values were reported by multiplying by a dilution factor of 20,000.

**AFP and PIVKA-II assays**

AFP was measured using the Tosoh AIA-600 II analyzer (Tosoh Bioscience, San Francisco, CA, USA) according to the manufacturer’s instructions. PIVKA-II was measured using a prototype chemiluminescent immunoassay (under development by Abbott Japan, Tokyo, Japan), which used paramagnetic microparticles coated with an antibody specific to PIVKA-II and an acridinium-labeled antibody specific to human prothrombin.

**Statistical analysis**

Descriptive statistics for GP73 and Fuc-HPX in the sample groups were compared using dot plots. The difference between group means was tested using the Kruskal-Wallis method. A p < 0.01 was used to determine statistical significance. The diagnostic accuracy of GP73, Fuc-HPX, AFP, and PIVKA-II assays for HCC and/or cirrhosis was evaluated using receiver operating characteristic (ROC) curve analysis, reporting the area under the curve (AUC) and its confidence interval (CI). The diagnostic cut-off for each test was determined using the Youden index (23). The analyses were performed using Analyse-it version 2.20 (Analyse-it Software, Leeds, UK) and SAS version 9.1 (SAS Institute, Cary, NC, USA).

**Results**

Dot plots comparing the values of GP73, Fuc-HPX, AFP, and PIVKA-II for a total of 229 samples from HCC, cirrhosis, hepatitis, and normal are shown in Figure 1. Three samples from patients undergoing Warfarin treatment were excluded from all analyses since Warfarin may lead to false increases in PIVKA-II concentrations. Total HPX concentrations were not evaluated for these sample groups, because no significant differences in total HPX distribution were observed for the groups with HCC, liver disease, and normals, as discussed later in this section. The values of the four markers by disease type are shown in Table 1. The distribution of GP73 concentrations in the cirrhosis samples was significantly higher than that seen in the hepatitis samples and in normals (p < 0.0001). The distribution of GP73 values in the HCC samples was not significantly different from that in the cirrhosis group (p < 0.4121). Fuc-HPX in the cirrhosis samples showed a significantly higher S/N distribution than that in samples from patients with hepatitis and normals (p < 0.0019 and p < 0.0001, respectively). There was no significant difference in the S/N distribution between the samples from patients with cirrhosis and HCC. The distributions of AFP and PIVKA-II in the HCC samples were significantly higher (p < 0.0001) than those seen in samples from patients with cirrhosis, hepatitis, and normals. The distribution of AFP in the HCC samples was significantly higher (p < 0.0001) than that seen in samples from patients with cirrhosis, hepatitis, and normals, while there was no significant difference in AFP in the samples from patients with cirrhosis, hepatitis, and normals. The distribution of PIVKA-II in the HCC samples was significantly higher (p < 0.0001) than that seen in samples from patients with cirrhosis, hepatitis, and normals.

GP73 and Fuc-HPX values from the three sample groups with HCC, cirrhosis, and hepatitis were analyzed by comparing HBV and HCV infection status. The dot plots are shown in Figure 2. The GP73 concentration in the HCV infected cirrhosis group was significantly higher than that in the HCV infection group (p < 0.0001), while that in the cirrhosis group with HBV infection was not significantly different than that in the group with HBV infection (p < 0.3239). GP73 concentrations in HCC patients with HCV infection were higher (p < 0.0192) than those in HCC patients with HBV infection. There was no significant difference in the Fuc-HPX distribution among the sample categories. In addition, AFP and PIVKA-II values from the HCC, cirrhosis, and hepatitis samples were analyzed by comparing HBV and HCV infection status. There was no significant difference in the distribution of AFP and PIVKA-II when comparing the three sample groups according to HBV and HCV infection status. There were no significant differences in the distribution of the four biomarkers according to the underlying biochemical mechanism of HCV infection, HBV infection, alcoholic liver injury, and others, with the exception of the distribution of GP73 between the HCC samples with HCV infection and HBV infection (p < 0.0063), and between the HCC samples with HCV infection and all others (p < 0.0004).

A total of 130 commercial samples from patients with HCC (n = 39), liver disease (n = 20), and normal blood donors (n = 71) were analyzed using total HPX ELISA and Fuc-HPX CLISA. The resulting dot plots are shown in Figure 3. The median total HPX concentrations in the samples from patients with HCC, liver disease, and normals were 1.03 g/L, 1.04 g/L, and 1.00 g/L, respectively. There was no significant difference in the distribution of total HPX concentrations for the sample groups. Fuc-HPX in the HCC samples showed a significantly higher S/N distribution than that in samples from patients with liver disease and normals (p < 0.0121 and p < 0.0001, respectively).

The sensitivity and specificity of the HCC and/or cirrhosis samples for the four biomarkers were evaluated using ROC.
Figure 1  Dot plots of serum levels of (A) GP73, (B) Fuc-HPX, (C) AFP, and (D) PIVKA-II in patients with HCC, cirrhosis, hepatitis, and in normal individuals. The values of GP73 and Fuc-HPX are shown on a linear scale, while the values of AFP and PIVKA-II are shown on a log scale due to a wide range of reported concentrations. The solid and dotted lines represent the mean and ±1 SD, respectively.

dot plot analysis. The ROC curves of the four markers are shown in Figure 4. For the combination of cirrhosis and HCC samples, GP73 showed the largest AUC of 0.90 (95% CI, 0.86–0.94) among the four markers, with a sensitivity of 91.3% and a specificity of 76.2% using a cut-off of 94.0 μg/L. Fuc-HPX showed an AUC of 0.77 (95% CI, 0.71–0.83), with a sensitivity of 61.5% and a specificity of 83.6% using a S/N cut-off of 1.91. AFP showed an AUC of 0.74 (95% CI, 0.67–0.80) with a sensitivity of 48.1% and a specificity of 95.1% using a cut-off of 15.3 μg/L. PIVKA-II

Table 1  Concentrations of the four serum markers in patients with HCC, cirrhosis, hepatitis, and in normal individuals.

<table>
<thead>
<tr>
<th></th>
<th>HCC (n = 70)</th>
<th>Cirrhosis (n = 34)</th>
<th>Hepatitis (n = 51)</th>
<th>Normal (n = 71)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Median</td>
<td>Mean ± SD</td>
<td>Median</td>
</tr>
<tr>
<td>GP73, μg/L</td>
<td>245 ± 183.1</td>
<td>177</td>
<td>298 ± 211.0</td>
<td>247</td>
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<td>Fuc-HPX, S/N</td>
<td>2.85 ± 1.96</td>
<td>2.35</td>
<td>2.78 ± 1.68</td>
<td>2.81</td>
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<tr>
<td>AFP, μg/L</td>
<td>9853 ± 41313.3</td>
<td>127.3</td>
<td>16.8 ± 48.6</td>
<td>4.1</td>
</tr>
<tr>
<td>PIVKA-II, AU/L</td>
<td>32942 ± 138308.2</td>
<td>720</td>
<td>552.4 ± 1827.2</td>
<td>67.3</td>
</tr>
</tbody>
</table>
Figure 2  Dot plots of serum levels of GP73 for HCC, cirrhosis, and hepatitis by (A) HCV infection and (B) HBV infection. The solid and dotted lines represent the mean and ±1 SD, respectively.

Figure 3  Dot plots of the serum levels of (A) total HPX and (B) Fuc-HPX in commercially obtained samples from patients with HCC, liver disease, and from normal individuals. The solid and dotted lines represent the mean and ±1 SD, respectively.

showed an AUC of 0.88 (95% CI, 0.83–0.93) with a sensitivity of 77.9% and a specificity of 95.1% using a cut-off of 57.7 AU/L. For HCC alone, GP73 showed an AUC of 0.78 (95% CI, 0.72–0.84) with a sensitivity of 88.6% and a specificity of 61.5% using a cut-off of 94.7 μg/L. Fuc-HPX showed the smallest AUC of 0.72 (95% CI, 0.65–0.79) among the four assays, with a sensitivity of 78.6% and a specificity of 60.9% using a S/N cut-off of 1.55. PIVKA-II showed the largest AUC of 0.90 (95% CI, 0.85–0.95), with a sensitivity of 84.3% and a specificity of 88.1% using a cut-off of 123.9 AU/L. AFP showed the second highest AUC of 0.81 (95% CI, 0.73–0.88) for HCC samples in this study, with a sensitivity of 62.9% and a specificity of 92.3% using a cut-off of 15.3 μg/L.

Discussion

HCC is the fifth most common cancer in men and the eighth most common cancer in women worldwide. Liver cirrhosis is the most important risk factor in the development of HCC. A combination of AFP testing in serum and ultrasound is widely used in the surveillance of patients with cirrhosis for...
HCC. In Japan, other tumor markers, such as PIVKA-II and AFP-L3, are also used for a screening diagnosis and/or monitoring. GP73 has been reported as a potential serum marker for the diagnosis of early HCC. Fucosylated proteins, such as fucosylated kininogen, fucosylated α-1-antitrypsin, and Fuc-HPX, show potential utility for liver disease, including HCC. There have been no reports that directly compared the sensitivity and specificity of GP73, fucosylated proteins, AFP, and PIVKA-II using samples from patients with HCC, cirrhosis, hepatitis, and healthy individuals. In this study, GP73 ELISA and Fuc-HPX CLISA were developed and evaluated for their diagnostic accuracy for HCC and/or cirrhosis, and compared with AFP and PIVKA-II.

The distribution of GP73 in patients with cirrhosis or HCC was significantly higher than that seen in patients with hepatitis and in normals, and had the largest AUC of 0.90 among the four markers used in this study. PIVKA-II had the largest AUC for HCC patients, followed by AFP. Some previous studies have demonstrated that GP73 is a potential marker for HCC by differentiating samples from patients with cirrhosis (10, 11), and another study has reported that GP73 is a potential marker for liver diseases and HCC (12). The results of our study are similar to those in the latter study. A recent study has demonstrated that PIVKA-II has greater accuracy for HCC than AFP, but the differences were not statistically significant (24). Another study has reported that AFP showed a greater AUC compared with PIVKA-II for early HCC (25). The results of diagnostic accuracy for PIVKA-II and AFP for HCC in this study are similar to those observed in the former study (24).

Samples in HCV infected patients with cirrhosis had significantly higher GP73 values than in HCV infected patients without cirrhosis. There may be a potential utility for GP73 in the diagnosis of cirrhosis, especially for the HCV infected high-risk group. Little is known about GP73 expression in samples from patients with liver diseases, but it can be suggested that its expression mechanism may be involved in the host response to viral infection or in the response to cytokines (26, 27). As the risk for the 5 year cumulative incidence of HCC is higher by a factor of approximately two in patients with HCV-related cirrhosis compared with those with HBV-related cirrhosis (4). GP73 may be useful in the detection of the early stage of progression of liver disease in HCV-infected patients.

The distribution of Fuc-HPX in commercially obtained samples from patients with HCC were significantly higher than that seen in samples from normal individuals (p < 0.0001). The distribution of total HPX concentrations did not show any statistical difference in the samples from normal individuals and from patients with liver disease and HCC. These results demonstrate that it is not the total HPX protein concentration, but the degree of fucosylation of HPX that is associated with HCC. An increased value of Fuc-HPX was observed in serum from patients with HCC, but was not observed in normal individuals in the proteomic analysis of a serum associated fucosylated glycoprotein (15), and in an immunoblot analysis (17). Our results for HCC patients and normal individuals were similar to the results observed in proteomic analysis and immunoblot analysis. However, the diagnostic performance of Fuc-HPX for HCC was inferior to that of the other existing markers, AFP and PIVKA-II. There was no significant difference in distribution of Fuc-HPX between alcoholic liver injury and viral hepatitis in this study. However, impaired glycosylation due to chronic alcohol abuse and deficient glycosylation to the specific protein need to be considered during the development of a marker of glycosylation.

In conclusion, we developed an enzyme-linked immunosorbent assay to determine GP73 concentrations in serum quantitatively, and a chemiluminescence-linked immunosorbent qualitative assay to detect Fuc-HPX using AAL. We evaluated the sensitivity and specificity of these two potential markers for diagnosing HCC and/or cirrhosis compared with AFP and PIVKA-II. GP73 showed a strong correlation with cirrhosis and HCC, and also showed a relationship to HCV infection. In addition, Fuc-HPX showed a correlation with HCC, but its accuracy for HCC was inferior to that of other assays. PIVKA-II, followed by AFP, showed the highest
sensitivity and specificity for HCC compared with the other markers examined in this study.

The data in this study need to be confirmed in a larger cohort of patients with liver disease to determine if GP73 in serum can act as a marker for the progression of liver disease. Future studies should evaluate samples from patients with progressive stages of fibrosis, cirrhosis, and HCC, and compare the results with those of other markers of liver disease.

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**Conflict of interest statement**

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