Diagnostic Value of the Hemopexin N-Glycan Profile in Hepatocellular Carcinoma Patients

Evi N. Debruyne,1 Dieter Vanderschaeghe,1,2 Hans Van Vlierberghe,3 Annelies Vanhecke,2 Nico Callewaert,2,4 and Joris R. Delanghe1*

BACKGROUND: Hepatocellular carcinoma (HCC) is a common and rapidly fatal cancer. Current diagnostic methods for HCC have poor sensitivity and specificity, are invasive, and carry risk for complications. Newer markers are needed to overcome these problems and allow diagnosis of HCC at an earlier stage. In view of known associations between glycosylation changes and liver disease, we focused on the serum glycoprotein hemopexin and the specific characteristics of this liver-synthesized glycoprotein.

METHODS: We studied 49 healthy volunteers and 81 patients divided into the categories of fibrosis, cirrhosis, and HCC with cirrhosis. Hemopexin was purified from study participants’ serum by use of heme agarose beads. The hemopexin N-glycan profile was determined by use of the DNA sequencer–assisted fluorophore-assisted carbohydrate electrophoresis technique.

RESULTS: We found that branching α-1,3-fucosylated multiantennary glycans on hemopexin were increased in the HCC group compared with the cirrhosis without HCC, fibrosis, and healthy volunteer groups, whereas nonmodified biantennary glycans decreased progressively across groups from fibrosis to the cirrhosis and HCC groups. Summarization of this information in a new marker, called the hemopexin glycan marker, enabled distinction of patients with HCC and cirrhosis from healthy volunteers and patients with fibrosis or cirrhosis with a sensitivity and specificity of 79% and 93%, respectively.

CONCLUSIONS: This study demonstrated hemopexin to be a model protein for studying liver-specific N-glycosylation. The hemopexin glycan marker could be a valuable complementary test to α-fetoprotein measurements for detection of HCC in patients with cirrhosis. Additional study of its utility for diagnosis and follow-up is recommended.

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Hepatocellular carcinoma (HCC) is a common cancer with a 5-year survival rate of 6%. Over half a million new cases are diagnosed each year. HCC, which often develops on a background of a cirrhotic liver, is frequently not diagnosed until it has reached an advanced stage when the remaining therapeutic options are less effective, leaving this disease with a poor prognosis (1, 2). However, surveillance of at-risk patients has proven beneficial. Surveillance improves detection and potentially curative treatment of small tumors. Reduction in mortality rates and the inequality in outcomes between patients diagnosed with early HCC and those with advanced HCC supports the application of screening (3). Currently used screening tools for HCC in patients with cirrhosis include ultrasonography and measurement of α-fetoprotein (AFP) concentrations in serum at a 6-month interval (4).

Suspicious screening results require further investigations according to established guidelines (4, 5). Several tools are available for diagnostic confirmation of a suspicious screening result. Depending on the size and features of the liver nodule found, the following techniques are used: ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), and as a last resort, liver biopsy. However, diagnostic accuracy of the radiological tools (CT and MRI) is tumor-size dependent and expensive (3–5), whereas biopsy is invasive and shows intra- or interobserver variability, sampling error, and risk of complications (6, 7).

1 Department of Clinical Chemistry, Microbiology and Immunology and 4 Department of Biochemistry, Physiology and Microbiology, Ghent University, Ghent, Belgium; 2 Unit for Molecular Glycobiology, Department for Molecular Biomedical Research, VIB, Ghent, Belgium; 3 Department of Gastroenterology and Hepatology, Ghent University Hospital, Ghent, Belgium.

* Address correspondence to this author at: Department of Clinical Chemistry, Ghent University Hospital, De Pintelaan 185, 8000 Gent, Belgium. Fax +32-9-332-36-59; e-mail joris.delanghe@ugent.be.

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5 Nonstandard abbreviations: HCC, hepatocellular carcinoma; AFP, α-fetoprotein; CT, computed tomography; MRI, magnetic resonance imaging; GnT, N-acetylgalactosaminyltransferase; DSA-FACE, DNA sequencer–assisted fluorophore-assisted carbohydrate electrophoresis. HBV, hepatitis B virus; Hpx, hemopexin; HA, heme agarose; NAJFcb, core-α-1,6-branching-α-1,3-fucosylated triantennary glycan; NA4Fb, branching-α-1,3-fucosylated tetra-antennary glycan; NA2, bigalacto biantennary glycan; CRP, C-reactive protein; MELD, Model for End-Stage Liver Disease; HCV, hepatitis C virus.
Therefore, finding newer markers for screening and diagnosing HCC at an early stage is of utmost importance.

Because glycosylation changes correlate with liver disease (8) and owing to the easy accessibility of serum, we focused our search for a new diagnostic marker on serum glycoproteins. Glycosylation is involved in both physiological and pathological events, such as cell growth, migration, differentiation, and tumor invasion. Glycosyltransferases, such as N-acetylgalactosaminyltransferase III (GnT-III), GnT-V, and α-1,6-fucosyltransferase, play a key role in this enzymatic process and result in the formation of branched N-glycans (9). The association between branched structures and various biological functions, including cell adhesion and cancer metastasis, is well known (9).

Using the sensitive DNA sequencer-assisted fluorophore-assisted carbohydrate electrophoresis (DSA-FACE) technique (10, 11) on total serum glycoprotein, it has recently been shown that the serum N-glycome yields a biomarker (named GlycoHCC-Test) that aids in the diagnosis of HCC in hepatitis B virus (HBV)-infected patients with cirrhosis. The GlycoHCC-Test enables the differentiation of HBV-infected patients with HCC from cirrhosis patients, with an overall sensitivity (57%) and specificity (88%) similar to that of serum AFP (12). However, serum glycoproteins represent a large group of glycoproteins synthesized by both the liver and plasma cells. We wanted to study the liver-specific N-glycan changes, so we selected hemopexin (Hpx), a 60-kDa heme-binding glycoprotein, because of several interesting characteristics. Serum concentrations of Hpx range from 0.4 to 1.5 g/L, which facilitates purification. The glycoprotein is mainly produced by the hepatocyte, cleared by the hepatocyte-specific membrane receptor, and recycled (13). It carries 5 N-glycans on its N-terminus and is a type II acute phase protein (13, 14). Finally, Hpx has no polymorphisms and does not bind any other plasma protein (13). These characteristics make this glycoprotein an ideal candidate to represent the liver-specific glycoproteome and possibly liver-specific changes.

We have explored the diagnostic possibilities of Hpx N-glycosylation changes as a marker for HCC, using the sensitive DSA-FACE technique (10, 11).

Materials and Methods

Patients

Serum samples from healthy volunteers (n = 49, 42.9% male) and liver disease patients (n = 81, 69.1% male) were collected from an outpatient clinic (Ghent University Hospital). Informed consent was given by all patients and the protocol was approved by Ghent University Hospital’s Ethics Committee. We divided patients into the following 3 categories: (1) HCC patients with a cirrhotic background (n = 20); (2) cirrhosis (n = 21); and (3) fibrosis patients (n = 40; F0, n = 10; F1, n = 10; F2, n = 10; F3, n = 10) without HCC. The median (interquartile range) ages in the patient and reference group were 59.0 (46.5–65.0) and 27.0 (24.5–35.0) years, respectively. We determined the diagnoses of the patients according to a combination of data (clinical, laboratory and imaging findings, and/or biopsy). The patients’ most relevant clinical data are summarized in Table 1.

Purification of hemopexin

We purified hemopexin using heme agarose (HA) beads (Sigma-Aldrich) (15). Briefly, 500 μL serum was incubated for 24 h with 400 μL HA beads at 4 °C. After centrifugation, supernatant was removed and the HA beads were extensively washed with 500 μL PBS (pH 7.5) to dispose of unbound proteins. Bound proteins were eluted at room temperature after incubation for at least 2 h with 500 μL of 0.2 mol/L sodium citrate elution buffer (pH 4), followed by overnight buffer exchange toward PBS (pH 7.5) to a final volume of 500 μL.

IgG depletion

Because IgG is the most abundant glycoprotein in serum (8–16 g/L), we performed additional IgG depletion through affinity chromatography with protein A agarose (GE Healthcare) on the Hpx purified fractions to dispose of remaining traces of IgG. We incubated 40 μL protein A agarose solution (binding capacity, 20 mg human IgG/mL) with 500 μL purified Hpx fraction. The mixture was shaken at room temperature for at least 1 h and subsequently loaded on a prewetted (MilliQ water) 96-well filtration plate containing a 0.45 μm hydrophilic Durapore® membrane (Millipore). After centrifugation, the flowthrough fraction was collected and used for further analysis. We repeated the same procedure of IgG depletion on total serum for each corresponding serum sample.

Concentration and purity assessment

We assessed the purity of the Hpx-purified solution by separation on 10% SDS-PAGE according to Laemmli (16), followed by Coomassie stain. Next, we performed Western blotting to identify the obtained protein as Hpx using a primary polyclonal rabbit anti-Hpx antibody (Siemens) and a peroxidase-conjugated secondary polyclonal sheep antirabbit antibody (The Binding Site). To prove absence of traces of other glycoproteins in the Hpx-purified fraction, we also performed
Western blotting against the most abundant glycoproteins: IgG, IgA, IgM, transferrin, haptoglobin, and α1-antitrypsin.

We used immunonephelometry on a BN Nephe- lometer II analyzer (Siemens) to measure the Hpx and IgG concentration pre- and post-Hpx purification and to confirm absence of IgG in the Hpx-purified fractions.

HEMOPEXIN N-GLYCAN ANALYSIS

Hpx N-glycans in the Hpx purified solution were released using the on-membrane deglycosylation method and labeled [11]. We analyzed labeled N-glycans by DSA-FACE with a capillary electrophoresis-based ABI3130 sequencer [11]. The peaks were further analyzed with GeneMapper version 3.7 software (Applied Biosystems). The peak height intensities were normalized to the total intensity of the measured peaks. Total serum and IgG-depleted serum from each corresponding sample were simultaneously analyzed.

STATISTICAL ANALYSIS

Statistical analyses were performed with SPSS 15.0 for Windows software (SPSS). The Mann–Whitney U-test was performed to search for variables that were significantly different between cirrhosis and HCC group. Significantly different variables between the groups were further analyzed by multivariate analyses using multiple linear regression.

We compared the different patient groups for the Hpx glycan marker using the Kruskal–Wallis test followed by a Dunn’s test. We used ROC curve analysis to compare the overall performance of the discussed markers.

Results

ISOLATION AND PURITY ASSESSMENT OF HEMOPEXIN FROM HUMAN SERUM

For the Hpx purification, we used affinity chromatography with HA beads based on the high affinity of Hpx toward heme (KDa <1 pmol/L). Mean concentration of Hpx after purification and additional IgG depletion was 0.02 g/L [yield 2.7% (2.4%) purified Hpx].

A Coomassie-stained SDS-PAGE was performed to prove purity of the Hpx-purified fractions. The gel...
ALTERATIONS IN THE N-GLYCAN PROFILE OF HEMOPEXIN

Using the highly sensitive and robust DSA-FACE technique (11), we analyzed N-glycan profiles from desialylated samples of control and patient group. We simultaneously analyzed serum, IgG-depleted serum, and Hpx-purified fractions. Because IgG is the major representative of the plasma cell–produced glycoproteome, a similar N-glycan profile of IgG-depleted serum and the Hpx-purified fraction would support our hypothesis that Hpx is a good representative for the liver-specific glycoproteome. Indeed, peaks specifically present on IgG (the undergalactosylated biantennary glycans) (17) were absent in both the IgG-depleted fractions and the Hpx-purified fractions (Fig. 2).

Each peak was quantified by normalizing its height to the sum of heights of all the measured peaks, followed by comparison of all peaks between control group, fibrosis patients, cirrhosis patients, and patients with cirrhosis and HCC. For the structural characterization and numbering of the peaks, we refer to Fig. 2.

We searched for glycan structures of Hpx that were increased or decreased in patients with HCC and cirrhosis compared to cirrhosis without HCC, fibrosis, and healthy volunteers to enable specific detection of HCC on a cirrhotic background. Although several Hpx peaks showed an increasing or decreasing trend toward HCC, numerical and graphical description of each individual peak revealed that the most relevant Hpx peaks to discriminate between cirrhosis patients with and without HCC were peak 5 (P < 0.01), peak 10 (P < 0.05), and peak 12 (P < 0.01) (see Supplemental File 1 and Supplemental Figs. 1 and 2, which accompany the
online version of this article at http://www.clinchem.org/content/vol56/issue5). Peak 12, a branching-α-1,3-fucosylated tetra-antennary glycan (NA4Fb), was rarely present in the control and fibrosis groups and increased from cirrhosis to HCC. Peak 10, a core-α-1,6-branching-α-1,3-fucosylated triantennary glycan (NA3Fcb), showed a rising pattern from healthy controls through fibrosis and cirrhosis to HCC (see online Supplemental Fig. 3). On the other hand, peak 5, a bigalacto biantennary glycan (NA2), showed a decreasing pattern from fibrosis through cirrhosis to HCC. The glycan structures of these 3 peaks are presented in Fig. 2.
To summarize the information gathered in the 3 Hpx peaks, we took the ratio of the sum of the increasing peaks 10 and 12 to the decreasing peak 5 \[
\frac{(P_{10}+P_{12})}{P_{5}}
\]. This combination of peaks was significantly increased in patients with HCC compared to cirrhosis patients \((P < 0.001)\), fibrosis patients, and healthy controls \((P < 0.001)\) (Fig. 3). We coined this new derived diagnostic variable \[(P_{10}+P_{12})/P_{5}\] the Hpx glycan marker for HCC.

\section*{Hpx GLYCAN MARKER AS A TUMOR MARKER FOR HCC}

Before claiming the Hpx glycan marker as a marker for HCC, we had to determine if it was a tumor marker and not a marker for liver failure. Furthermore, correction for the parameters [age, AFP, albumin, and C-reactive protein (CRP); see Table 1] showing significant difference between cirrhosis and HCC plus cirrhosis was necessary.

There is no correlation between MELD (Model for End-Stage Liver Disease) score, a marker of risk of death that reflects the severity of underlying chronic liver disease, and the Hpx glycan marker. Additionally, in contrast to the Hpx glycan marker, no significant difference could be demonstrated by the Mann–Whitney \(U\)-test between cirrhosis patients and patients with HCC plus cirrhosis for the MELD score.

Multivariate analysis showed there was still a significant difference \((P < 0.05)\) between cirrhosis and HCC plus cirrhosis for the Hpx glycan marker, even after adding the parameters age, AFP, albumin, and CRP to the model. There is also a significant difference in sex distribution between healthy volunteers and patient group \((P < 0.01)\), but Spearman correlation testing demonstrated that there was no correlation between sex and individual N-glycans or between sex and the Hpx glycan marker. Tumors of HCC patients were staged using the Milan criteria. No correlation was found between the Hpx glycan marker and tumor stage.

\section*{EFFICIENCY OF THE Hpx GLYCAN MARKER AS A HCC TUMOR MARKER}

Although AFP remains the preferred serum tumor marker for screening and diagnosing HCC, the value of AFP as a marker for HCC has been disputed owing to its relatively poor sensitivity and specificity \((18)\). As shown in Fig. 4A by ROC analysis, our Hpx glycan marker \[(P_{10}+P_{12})/P_{5}\] for HCC was able to discriminate HCC patients from non-HCC patients with an accuracy of 92\% (3\%), which exceeds the diagnostic accuracy of 82\% (7\%) of the conventionally used AFP marker. At the cutoff value of 0.014, the Hpx glycan marker for HCC showed a sensitivity of 79\% and a specificity of 93\%. Such diagnostic efficiency could not be reached by AFP in our study.

Fig. 4B demonstrates that when narrowing the clinical question to screening for HCC in cirrhosis patients, the diagnostic accuracy of our Hpx glycan marker for HCC changed to 82\% (7\%), with a sensitivity of 60\% and a specificity of 85\% at a cutoff of 0.016. The diagnostic accuracy for AFP in this context was found to be 74\% (8\%). Combining the Hpx glycan marker with AFP demonstrated its added value to AFP for detecting HCC in patients with cirrhosis (Fig. 5). In this same narrowed patient setting, the diagnostic accuracy for the GlycoHCCtest was 62\% (9\%), with a sensitivity and specificity of 50\% and 70\%, respectively, at the published cutoff of \(-0.34\) \((12)\).

\section*{Discussion}

We coined the name “Hpx glycan marker” for a test that combined information from peaks 5, 10, and 12 of the glycan profile from each individual as \[(P_{10}+P_{12})/P_{5}\]. Results for this marker enabled us to discriminate patients with cirrhosis and HCC from patients with cirrhosis without HCC.
was selected because of its interesting characteristics, making it an ideal candidate to represent the liver-specific N-glycome.

Using the sensitive and robust DSA-FACE technique (10, 11), the core-α-1,6-branched-α-1,3-fucosylated triantennary glycan (NA3Fcb or peak 10) and the branching-α-1,3-fucosylated tetra-antennary glycan (NA4Fb or peak 12) were more abundant on Hpx in HCC patients than in cirrhosis or fibrosis patients. N-acetylglucosaminyltransferase V (GnT-V), which catalyzes the formation of these multiantennary structures via the addition of a β-1,6-GlcNAc residue, is strongly upregulated in HCC and associated with progression of HCC (9, 19, 20). These multiantennary N-glycans are easily modified with an α-1,3 branch fucose, producing NA3Fcb (peak 10) and NA3Fb (peak 12). This is in agreement with previous studies that have confirmed the increased activity of the α-1,3-fucosyltransferase and its correlation with various cancers (9, 19). At this moment, however, we cannot exclude the possibility that peaks 10 and 12 are also partially the result of GnT-IV. Combining these findings explains the increase of NA3Fcb (peak 10) and NA4Fb (peak 12) in our HCC patients. As a result of this branching, we observed a progressive decrease of the bigalacto biantennary glycan (NA2 or peak 5).

The information enclosed in those 3 peaks was combined into a new marker (the Hpx glycan marker) by taking the ratio of the fucosylated multiantennary peaks 10 and 12 (respectively NA3Fcb and NA4b) to the nonfucosylated biantennary peak 5 (NA2).

The Hpx glycan marker allowed HCC patients with a cirrhotic background to be distinguished from patients with cirrhosis or fibrosis and healthy volunteers with a diagnostic accuracy of 92% and sensitivity and specificity of 79% and 93%, respectively, at a cutoff value of 0.014. Such diagnostic efficiencies could not be demonstrated for serum AFP in our patients, nor have they previously been described (18). In our study group, the diagnostic performance of the conventionally used serum AFP marker was found to be 82%. The major drawback of AFP as diagnostic marker for HCC was its poor sensitivity and specificity (18). Despite the established correlation between AFP and HCC, increased serum AFP concentrations also have been associated with several other types of cancer, chronic...
hepatitis, and liver cirrhosis (18, 20–23). In this study, a comparable specificity of AFP for detecting HCC was reached at a cutoff of 10 µg/L, but sensitivity was only 52%. At this threshold, approximately half of HCC cases would be missed. Lowering the cutoff identified more cases at the expense of specificity.

Independence of MELD score and clinical chemistry parameters established the Hpx glycan marker as a valid tumor marker. Although there is a significant difference in sex distribution between healthy controls and patients, we did not correct for this factor in the model. This could be considered a potential study weakness. However, there was no correlation between sex and the Hpx glycan marker, suggesting that the Hpx glycan marker adds value to AFP in diagnosing HCC in cirrhosis patients. (AFP values underwent logarithmic transformation.)

Comparing the Hpx glycan marker with the GlycoHCC test in this narrowed patient setting, the diagnostic accuracy of the GlycoHCC test was found to be 62% (9%), with sensitivity of 50% and specificity of 70% at the published cutoff of −0.34 (12). This is worse than both the Hpx glycan marker and AFP. The GlycoHCC test appeared to be less informative in diagnosing HCC in cirrhotic chronic hepatitis C virus (HCV) patients than in the original work described by Liu et al. (12), which posed the same question for cirrhotic chronic HBV patients.

A possible explanation for this poor diagnostic performance of the GlycoHCC test could be the etiologic background. The study population in the GlycoHCC test included exclusively HBV-infected patients (12), in contrast to our patient population consisting mainly of HCV patients. Such differences could be attributed to the different infection mechanism of these viruses—HCV is mainly an intracellular infection and HBV extracellular. HBV transfection influences glycosylation through its effect on the activity of glycosyltransferases, more specifically on GnT-III (24, 25). It is possible that the serum protein N-glycome alterations reflected in the GlycoHCC test are specifically an outcome of HBV infection. Our study included no HBV-infected patients, and this could explain why these serum protein N-glycomic changes of the GlycoHCC test were not observed in our study.

In conclusion, we introduce the Hpx glycan marker as a new tumor marker for the detection of HCC in HCV cirrhosis patients. The Hpx glycan marker could be a valuable complementary test to the currently used conventional diagnostic methods for HCC in screening HCV cirrhosis patients. Further studies investigating larger study populations over longer periods will be required to examine whether specific glycosylation changes of Hpx are associated with specific liver disease etiologies.

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