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Mass-Selected Site-Specific Core-Fucosylation of Ceruloplasmin in Alcohol-Related Hepatocellular Carcinoma

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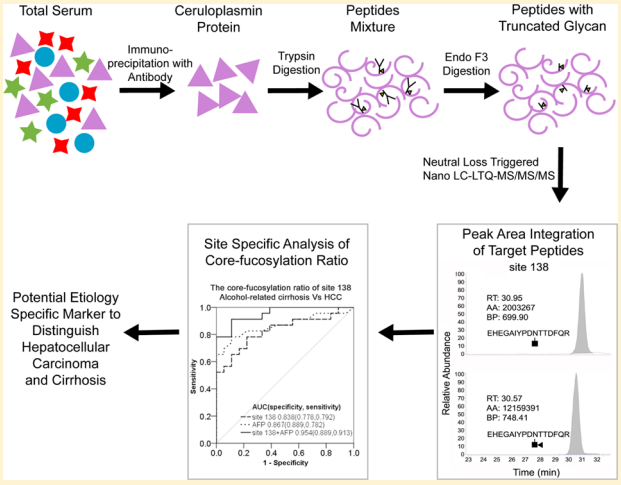
Supporting Information

ABSTRACT: A mass spectrometry-based methodology has been developed to study changes in core-fucosylation of serum ceruloplasmin that are site-specific between cirrhosis and hepatocellular carcinoma (HCC). The serum samples studied for these changes were from patients affected by cirrhosis or HCC with different etiologies, including alcohol, hepatitis B virus, or hepatitis C virus. The methods involved trypsin digestion of ceruloplasmin into peptides followed by Endo F3 digestion, which removed most of the glycan structure while retaining the innermost *N*-acetylglucosamine (GlcNAc) and/or core-fucose bound to the peptide. This procedure simplified the structures for further analysis by mass spectrometry, where four core-fucosylated sites (sites 138, 358, 397, and 762) were detected in ceruloplasmin. The core-fucosylation ratio of three of these sites increased significantly in alcohol-related HCC samples (sample size = 24) compared to that in alcohol-related cirrhosis samples (sample size = 18), with the highest AUC value of 0.838 at site 138. When combining the core-fucosylation ratio of site 138 in ceruloplasmin and the alpha-fetoprotein (AFP) value, the AUC value increased to 0.954 ($OR_{site138}$ = 12.26, p = 0.017; OR_{AFP} = 3.64, p = 0.022), which was markedly improved compared to that of AFP (AUC = 0.867) (LR test p = 0.0002) alone. However, in HBV- or HCV-related liver diseases, no significant site-specific change in core-fucosylation of ceruloplasmin was observed between HCC and cirrhosis.



KEYWORDS: *Core-fucosylation, hepatocellular carcinoma, ceruloplasmin, site specific*

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common malignancy and the third most common cause of cancer-related death worldwide.[1] HCC generally develops in patients that already have liver cirrhosis where this group needs to be monitored clinically in order to detect an early onset of HCC. HCC may develop in patients either with ALC (alcohol)-, hepatitis B virus (HBV)-, or hepatitis C virus (HCV)-related cirrhosis.

Effective treatments for HCC include tumor resection, liver transplantation, or percutaneous treatment. In the United States, Europe, and Japan, these treatments can be applied to only 30% of the patients (who are diagnosed at early stage), and they result in a 5 year survival rate higher than 50%. In comparison, for late stage patients where these treatments cannot be applied, the 2 year survival rate is lower than 16%.[2] Therefore, early detection of HCC is imperative.

Methods for noninvasive detection and monitoring of HCC include imaging (ultrasound, computer tomography, and magnetic resonance imaging) and serum marker analysis.[3] However, because the interpretation of imaging is operator-dependent and can be very difficult for persons who are obese, who have underlying cirrhosis, or who have small tumors, reliable serum markers are needed to complement these imaging procedures. Serum alpha-fetoprotein (AFP) is most widely used as a clinical HCC diagnostic marker because of its relatively high AUC value (area under the curve of ROC) of 0.82 between HCC and chronic liver disease samples; at the cutoff value of 20 ng/mL, it has a specificity of 90.6% and a sensitivity of 60.0%.[4] Des-gamma carboxy prothrombin (DCP) is widely used as a marker in Japan. The diagnostic value of DCP varies among different groups of patients. In patients with small (<3 cm) tumors, DCP has an AUC value of 0.732, lower than that of AFP (0.870); in patients with large (>5 cm) tumors, it has an AUC value of 0.985, higher than that of AFP (0.934).[5] In addition, in

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patients with viral etiology DCP, it has an AUC value of 0.76; in patients with nonviral etiology, it has an AUC value of 0.65.[6] A recent large clinical study has shown that AFP and DCP are complementary but neither is sufficiently accurate to be used for HCC surveillance.[7]

Hence, new diagnosis strategies are urgently needed for early HCC detection. Along these lines, core-fucosylation has recently been used as a potential marker for various cancers, including pancreatic cancer,[8] lung cancer,[9] and liver cancer.[10] AFP-L3, where *Lens culinaris* agglutinin (LCA) binds to the core-fucosylated glycopeptide of AFP, has been used as an alternative marker for HCC.[11] A commercial diagnostic kit for AFP-L3 is currently available in Japan (Wako Pure Chemical Industries Ltd., Amagasaki). Using this AFP-L3 kit for patients with AFP < 20 ng/mL, the AUC value between HCC and chronic liver disease was 0.707, with a specificity of 85.1% at a sensitivity of 41.5%.[12] In other work, the *Aleuria aurantia* lectin (AAL)-binding part of alpha-1-antitrypsin (A1AT), which comprises both core-fucosylated and antennary-fucosylated glycopeptides, was found to have an AUC value of 0.867 to distinguish HCC from cirrhosis samples with a specificity of 86% at a sensitivity of 70%. Further analysis showed that antennary-fucosylated A1AT gave more frequent false positives, whereas core-fucosylated A1AT did not, indicating that only the increase of core-fucosylated A1AT is specific for HCC.[13]

Data available in the literature indicate that ceruloplasmin is upregulated in various lung,[14] pancreatic,[15] colon/rectum,[16] and liver cancers.[17] In previous work, ceruloplasmin was found to be upregulated in LCA-enriched HCC serum samples, which indicates the upregulation of core-fucosylated ceruloplasmin.[13,17] However, it was not known whether the observed increase was common to HCC with various etiologies or unique to HCC with a specific etiology. Additionally, ceruloplasmin contains several glycosites, so it is unclear which sites contribute to the effect as well as the relative contribution of each site.

In this study, the site-specific core-fucosylation ratio of ceruloplasmin in patients with liver diseases (cirrhosis or HCC) of three major etiologies (ALC, HBV, or HCV) was analyzed using a mass spectrometry-based assay. Our study shows that there are four core-fucosylated sites in ceruloplasmin, among which the core-fucosylation ratio of sites 138 and 397 are more susceptible to change in disease samples. The most significant change among normal controls, ALC-related cirrhosis, and ALC-related HCC samples can be attributed to the core-fucosylation ratio of site 138 with an AUC value of 0.922 between normal and ALC-related cirrhosis and an AUC value of 0.838 between ALC-related cirrhosis and ALC-related HCC, whereas in patients with HBV or HCV etiology, no significant change was observed in cirrhosis samples versus HCC samples.

MATERIALS AND METHODS

Serum Samples

All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. Barcelona Clinical Liver Cancer (BCLC) staging system was used to divide HCC samples into two groups: samples at stage A were considered to be early stage and samples at stage B, C, or D were considered to be late stage. All of the serum samples were collected at the University Hospital of Michigan. The set of serum samples used in this study contained a total of 116 samples, which were composed of 15 normal controls, 18 ALC-related cirrhosis samples, 12 ALC-related early stage HCC samples, 12 ALC-related late stage HCC samples, 9 HBV-related cirrhosis samples, 6 HBV-related early stage HCC samples, 3 HBV-related late stage HCC samples, 18 HCV-related cirrhosis samples, 9 HCV-related early stage HCC samples, and 14 HCV-related late stage HCC samples. This study was approved by the Institutional Review Board of the University of Michigan Medical School. In North America, about 30−50% of HCC is related to HCV infection and about 10% is related to HBV infection.[18] Because of the limited access to HBV-related liver disease samples in the United States, only 9 HBV-related cirrhosis and 9 HBV-related HCC samples were included in this study. All of the serum samples were stored at −80 °C before use. A summary of the clinical data is given in Table 1.

Table 1. Clinical Indices of the Patient Serum Samples Used in This Study

disease group[a]	samples size	age (year) (median, range)	gender (F/M)
normal	15	59 (45−89)	4:11
ALC_cirrhosis	18	63 (48−70)	5:13
ALC_HCC (ES/LS)	24 (12:12)	66 (44−79)	5:19
HBV_cirrhosis	9	53 (37−62)	0:9
HBV_HCC (ES/LS)	9 (6:3)	54 (25−84)	0:9
HCV_cirrhosis	18	55 (48−78)	5:13
HCV_HCC (ES/LS)	23 (9:14)	57 (49−69)	6:17

[a]ES, early stage (BCLC staging, stage A); LS, late stage (BCLC staging, stage B, C, or D).

Immunoprecipitation

The procedure was performed following previously described methods[8] with some modifications. The brief workflow of this study is shown in Supporting Information Figure S1. Human serum (10 μL) was thawed on ice and diluted to 200 μL with coupling buffer (10 mM sodium phosphate, 150 mM sodium chloride, pH 7.2) using a Cross-link IP kit (Pierce Scientific, Rockford, IL). IgG is the most abundant glycoprotein in serum, with a concentration of 8−16 mg/mL, and has high affinity to protein A/G agarose beads. IgG will severely contaminate the immunoprecipitated target protein, so it was depleted prior to ceruloplasmin immunoprecipitation. In this procedure, diluted serum was incubated with 100 μL of protein A/G agarose slurry in a 900 μL spin column on an end-to-end rotator at 4 °C for 3 h. The depleted serum dilution was spun in a centrifuge at 1500*g* for 30 s. The beads were washed once with 100 μL of coupling buffer.

Immunoprecipitation was performed using the Cross-link IP kit according to the supplier's protocol. Monoclonal ceruloplasmin antibody (3 μg; Abcam, Cambridge, MA) was bound to 20 μL of protein A/G plus agarose slurry at room temperature for 30 min in 100 μL of binding buffer system and cross-linked with the beads by 9 μL of 2.5 mM disuccinmidyl suberate (DSS) cross-linker in 50 μL of binding buffer system at room temperature for 30 min. Unbound antibody that was not cross-linked was removed by extensive washing with coupling buffer followed by elution buffer (100 mM glycine-HCl, pH 2.8). The antibody-conjugated beads were then incubated with IgG-depleted serum at 4 °C overnight, and elution was carried out with 60 μL of elution buffer twice. The eluted ceruloplasmin was neutralized with 12 μL of Tris saline buffer (1 M Tris, 0.76 M NaCl, 0.052 M KCl, pH 8) to avoid precipitation in the following desalting step. It was then desalted with 500 μL Zeba Micro Spin Desalting columns 7K MWCO (Pierce Scientific, Rockford, IL), which

were prewashed 5 times with 300 μL of 100 mM TEAB. The desalted sample was dried in a SpeedVac concentrator (Labconco, Kansas City, MO) at room temperature and redissolved with 10 μL of 100 mM TEAB.

Trypsin and Endo F3 Double Digestion

Immunoprecipitated samples were reduced by 20 mM TCEP at 37 °C for 30 min and were then alkylated by 20 mM IAA at room temperature for 15 min. The samples were then diluted 3 times with 100 mM TEAB and digested with 0.8 μL of trypsin (0.5 μg/μL) at 37 °C overnight. Trypsin-digested samples were sonicated in an ice−water bath for 5 min and desalted with a C18 column (Pierce Scientific, Rockford, IL). The columns containing 8 mg of C18 resin, which can bind up to 30 μg of total peptide, were prewetted with 200 μL of 0.1% TFA in 50% acetonitrile 5 times and equilibrated with 0.1% TFA in water 3 times at 1500*g* for 0.5 min. The peptides were bound to the C18 medium 5 times followed by 3 times washing with 0.1% TFA to remove nonspecific binding at 1500*g* for 0.5 min. Twenty microliters of 50% acetonitrile with 0.1% TFA was used for elution at 1500*g* for 0.5 min. The elution was repeated twice, and the eluates were pooled for further use. Samples were dried in a SpeedVac concentrator (Labconco, Kansas City, MO) at room temperature. The desalted samples were reconstituted with 29.6 μL of Endo F3 buffer (50 mM sodium acetate, pH 4.5). Endo F3 was used to partially truncate the glycan structure of the glycopeptides. After Endo F3 digestion, only the innermost GlcNAc and/or the core-fucose remained attached to the asparagine residue of the peptide, which greatly simplifies the identification of the glycopeptides by mass spectrometry. The samples were pipetted well and sonicated in an ice−water bath for 5 min. Endo F3 (0.4 μL, 2 mU; QA-Bio, San Mateo, CA) was added for overnight digestion at 37 °C. Endo F3 digested samples were deactivated at 95 °C for 5 min, and TFA was added to make the final concentration 0.1%. After sonication in an ice−water bath for 5 min, the samples were desalted with a C18 column as described above. After evaporation in a SpeedVac concentrator (Labconco, Kansas City, MO) at room temperature, the samples were ready for mass spectrometry analysis. Half of the sample was loaded on an LC-LTQ-MS for one run.

Nano LC-LTQ-MS

Nano LC-MS/MS conditions were used as described in previous work.[8] In summary, a Magic C18 capillary column (100 μm i.d. × 15 cm; 3 μm particles, 200 Å) (Michrom Biosciences, Auburn, CA) was used for LC separation, and gradient elution was performed using a Paradigm MG4 micropump system (Michrom Biosciences, Auburn, CA) with a flow rate at 350 nL/min. Mobile phase A was 2% acetonitrile with 0.1% formic acid in water, and mobile phase B was 2% water with 0.1% formic acid in acetonitrile. The analytical gradient lasted for 80 min, where the composition of solvent B rose from 5 to 32% in 50 min followed by a washing and equilibration step where solvent B increased to 95% in 1 min, was held for 4 min, returned to 5% B in 0.1 min, and was held for 25 min.

An ESI-LTQ mass spectrometer (Thermo Fisher Scientific, San Jose, CA) operated in positive ion mode was used for analysis. The ESI spray voltage and capillary voltage were set at 2.2 kV and 45 V, respectively. Collision ionization dissociation (CID) fragmentation was performed at 35% of the normalized collision energy. The mass spectra were acquired in a data-dependent manner. Following a full scan in the mass range of m/z 400−1800, CID MS/MS was performed on the most intense ions, and the fragment ion in MS/MS with neutral loss of 73 or 48.67 (m/z) was selected for CID MS/MS/MS fragmentation. CID MS/MS and MS/MS/MS were performed on the first to the fourth most intense ions from the survey MS full scan. The default charge state was set at 3+ for MS2 and 2+ for MS3. The scan range of MS2 and MS3 is defined automatically by the LTQ depending on the default charge states and is dynamic for each MS2 or MS3 scan.

Database Search

The mass spectrum was searched with Proteome Discoverer 1.2 (Thermo Fisher Scientific, San Jose, CA) software with SEQUEST using the following settings: (1) fixed modification: cysteine carbamidomethylation (+57.0 Da); (2) dynamic modification: methionine oxidation (+16.0 Da), addition of GlcNAc (+203.1 Da), or GlcNAc+Fucose (+349.2 Da) to asparagine residue; (3) one missed cleavage was allowed; (4) peptide ion tolerance: 1.4 Da; (5) fragment ion tolerance: 0.8 Da; and (6) Swiss-Prot *Homo sapiens* database (release 2010_10, downloaded on Nov 2, 2010) was used.

The identified target peptides were quantified manually with Xcalibur Qual Browser 2.1 (Thermo Fisher Scientific, San Jose, CA) using the peak area from the extracted ion chromatogram (XIC) with the following settings: (1) precursor peaks were extracted with a 1 Da (±0.5 Da) mass window, (2) scan filter was set as full MS, (3) boxcar-type of smoothing with 7 points was enabled, and (4) Genesis peak detection algorithm was used.

Statistic Analysis

The core-fucosylation ratio of each site was calculated as the peak area of core-fucosylated peptides divided by that of non-core-fucosylated peptides. The core-fucosylation ratio of each site in different liver diseases was compared using GraphPad Prism 5, and power analysis was done with GraphPad StatMate 2 (GraphPad, La Jolla, CA). D'Agostino−Pearson omnibus normality test was used to check the distribution of core-fucosylation ratios of ceruloplasmin at each site before the following Student's *t*-test. The difference between means of the groups was analyzed using the *t*-test with Welch's correction (Gaussian distribution was assumed, and equal standard deviation was not assumed) at confidence intervals of 95% and two-tailed p values. For power analysis, the average standard deviation (SD) of each of the groups being compared was used, with the significance level of 0.05, two-tailed. For example, the core-fucosylation ratio of ALC-related cirrhosis at site 138 is 2.170 ± 0.614 (mean ± SD) and that of ALC-related HCC is 3.581 ± 1.424, so the average SD of the two groups (1.019) was used for sample size estimation. In order to differentiate the means of the two groups, the minimal sample size was 14 to reach 90% power. The sample sizes used in this study guaranteed that all of the significance analyses had statistical power larger than 90% for the observed effect size. The receiver operator characteristic (ROC) curve of the core-fucosylation ratio between two disease samples or normal control samples was generated with SPSS 13 (IBM, Armonk, NY). The ROC analysis of core-fucosylation ratio and AFP was performed as follows: (1) predicted value of probabilities was generated with binary logistic regression using core-fucosylation ratio and AFP as covariates and (2) the ROC curve was generated on the basis of the predicted probabilities. The AUC accuracy for the ROC curve of this combined analysis was checked by 10-fold cross-validation considering the relatively small sample size.

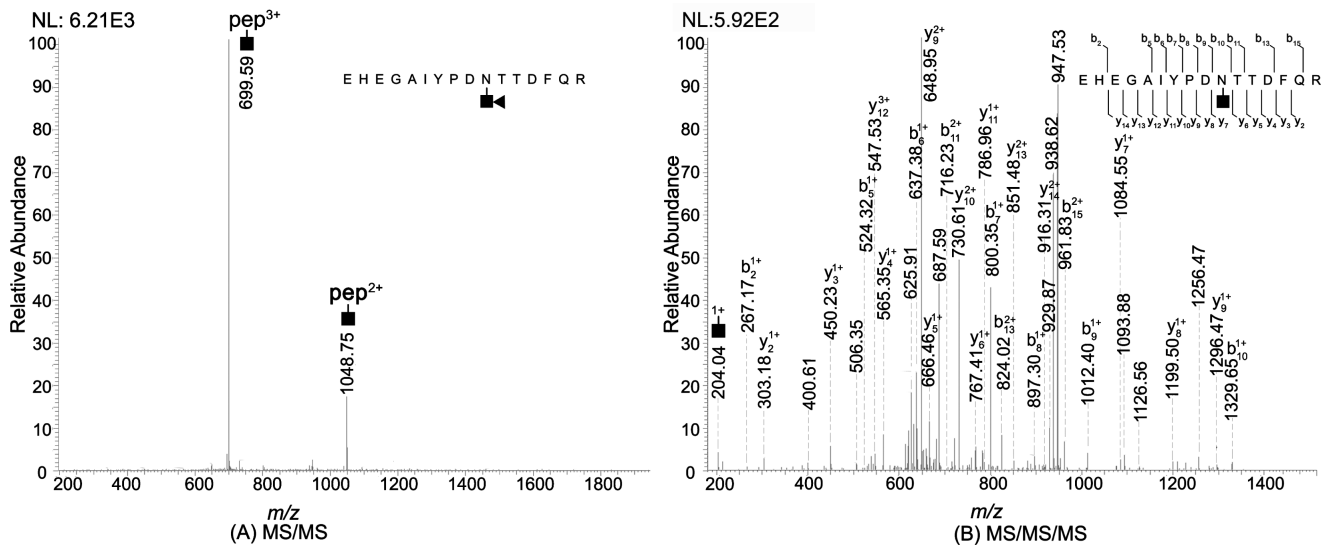

Figure 1. (A) MS/MS mass spectrum of the interested core-fucosylated parent ion peptide EHEGAIYPDnTTDFQR at m/z 748.46 with the neutral loss peak ion at m/z 699.8. (B) MS/MS/MS mass spectrum of the neutral loss peak ion from panel A. NL: peak intensity.

Table 2. Core-Fucosylated Sites of Ceruloplasmin

			without CoreF[b] (m/z)		with CoreF (m/z)	
glycosylated site	peptide sequence[a]	charge	theoretical	observed[c] (Xcorr)	theoretical	observed (Xcorr)
site 138	EHEGAIYPDnTTDFQR	3+	699.68	699.78 ± 0.18 (3.47)	748.37	748.40 ± 0.18 (2.62)
site 358	AGLQAFFQVQECnK	2+	922.45	922.46 ± 0.32 (3.45)	995.47	995.60 ± 0.27 (2.76)
site 397	EnLTAPGSDSAVFFEQGTTR	2+	1166.16	1165.96 ± 0.20 (4.01)	1239.19	1239.13 ± 0.24 (2.19)
site 762	ELHHLQEQnVSNAFLDK	3+	742.76	742.80 ± 0.20 (3.24)	791.45	791.53 ± 0.15 (2.47)

[a]Glycosylated asparagine residue is indicated by n in the peptide sequence. [b]CoreF: core-fucosylation. [c]Observed m/z is calculated as the mean ± SD of all identified glycopeptide spectra from five randomly chosen samples.

RESULTS AND DISCUSSION

It has been shown that the pathology of liver diseases with different etiologies, including ALC, HBV, and HCV, is very different.[19,20] Combining all cirrhosis or HCC samples with different etiologies together for analysis may mask potential markers for each type of liver disease. In this work, we have studied the core-fucosylation of serum ceruloplasmin as a marker of HCC, where the study focused specific investigation on each disease subtype. The selection of ceruloplasmin is based on a previous study[17] where using mass spectrometry and lectin blots it was found that some ceruloplasmin proteins that could bind to LCA were upregulated in HCC samples compared to those in cirrhosis samples. However, in that study, HCC with various etiologies was not analyzed separately. Furthermore, the overall result with lectin blots only revealed that the core-fucosylation ratio of the entire protein was changed without any indication of the possible contribution of each specific site and thus it may mask the core-fucosylation change at the site with the strongest response in certain disease states.

In this study, we have identified four core-fucosylated sites of ceruloplasmin and further analyzed the site-specific core-fucosylation ratio changes in liver cirrhosis and HCC with various etiologies, including ALC, HBV, and HCV, aiming to search for specific changes that can be used as markers of HCC.

Ceruloplasmin Core-Fucosylated Sites

Ceruloplasmin isolated from the serum sample was first digested into peptides with trypsin and was then digested with Endo F3. The sequence coverage of ceruloplasmin identified by MS was over 50%, as shown in Supporting Information Figure S2. The SDS-PAGE gel result of immunoprecipitated ceruloplasmin from two samples is shown in Figure S3, using 0.3 μg of commercially purified ceruloplasmin as control.

After Endo F3 digestion, only the innermost GlcNAc and/or the core-fucose remained attached to the asparagine residue of the peptide.[21] This is shown in Figure 1A, where the peptide EHEGAIYPDnTTDFQR with core-fucosylated site 138 had a mass of 748.46, with the charge of 2+ and 3+. Using MS/MS CID in the LTQ mass spectrometer, the core-fucose tended to be lost and to generate a neutral loss peak with a mass of m/z 699.8, a reduction of 146 Da (corresponding to the mass of fucose) from the parent ion. MS/MS/MS was used sequentially to fragment this neutral loss peak for peptide identification, as shown in Figure 1B. For peptides without core-fucosylation, they were readily fragmented in MS/MS.

According to the NXT/S motif (X is not proline) for N-glycosylation, there are seven potentially glycosylated sites in ceruloplasmin (sites 138, 227, 358, 397, 588, 762, and 926). Sites 227 and 926 were not detected in this experiment and have not been reported in previously published work.[22] Sites 138, 358, 397, 588, and 762 were detected in this experiment, but only sites 138, 358, 397, and 762 were glycosylated and/or core-fucosylated, as shown in Table 2. All four target core-fucosylated peptides were identified with the same method as described above. The MS/MS and MS/MS/MS spectra and the matched peak list of MS/MS/MS spectra with respective theoretical peaks are shown in Supporting Information Figure S4.

Charge 2+ and/or charge 3+ were detected in the target glycopeptides. However, only one of the four target glycopeptides (site 397) has two charge states in all of the tested samples.

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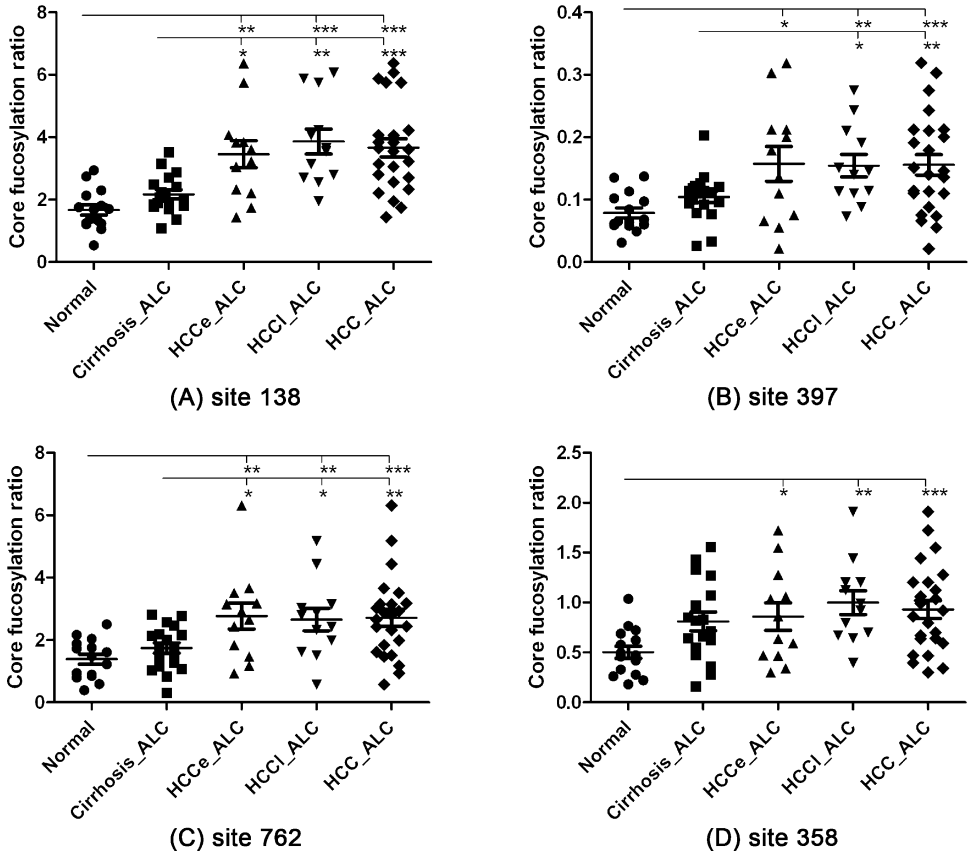

Figure 2. Core-fucosylation ratios of ceruloplasmin at sites 138 (A), 397 (B), 762 (C), and 358 (D) in normal and ALC-related serum samples. HCCe, HCC early stage; HCCl, HCC late stage; HCC, all HCC samples. A *t*-test comparison was made between each disease group and normal group as well as between cirrhosis and each disease group. Error bar indicates SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

The work of Stavenhagen[23] has shown that the intensities of one peptide with different charge states are different. We have also noticed this from our LTQ mass spectrometry result. In this study, the charge state with a stronger peak intensity that could be detected in all tested samples was chosen for each target peptide, as listed in Table 2, to determine the ROC value for distinguishing HCC from cirrhosis.

Core-Fucosylation Ratio: An Index of Core-Fucosylated Site in Ceruloplasmin

The intensity of the core-fucosylated peptide and the non-core-fucosylated peptide in MS1 was integrated, as shown in Figure S5, and the core-fucosylation ratio was calculated as follows:

$$\text{core-fucosylation ratio} = \frac{\text{area (XIC of core-fucosylated peptide)}}{\text{area (XIC of corresponding non-core-fucosylated peptide)}}$$

From Figure S5, it was also found that each core-fucosylated peptide eluted about 30 s before its related non-core-fucosylated peptide. This is probably due to the addition of core-fucose, which reduces its hydrophobicity.

The potential of false positives during integration is avoided by (1) immunoprecipitation, which simplifies the sample complexity analyzed by the LTQ, and (2) a careful check of the spectra of the target peptides based on the retention time, the m/z of the parent ion, and the MS2 and MS3 fragmentation profiles. The possibility of contamination of the target glycopeptides is thus exluded. Note that the intensity of these glycopeptides does not reflect the exact quantity of the peptides because of their different ionization efficiencies.[23] This index was used to make a comparison between different disease states rather than to provide the ratio of the exact quantity of the glycopeptides.

Site Preference in Core-Fucosylation of Ceruloplasmin

Each core-fucosylated site of ceruloplasmin appears to have a different response in HCC samples compared to that in normal controls. The core-fucosylation ratio in ALC-related HCC early stage and late stage samples was significantly higher than those of normal controls at all four sites (Figures 2 and 3), whereas in HBV-related HCC samples, the upregulation of core-fucosylation was found at site 397 (Figure 4) and at sites 138 and 397 in HCV-related HCC samples (Figure 5). Thus, all four sites in ceruloplasmin are sensitive to ALC-related HCC, whereas in HBV/HCV-related HCC samples, the core-fucosylation ratio at site 138 and/or site 397 are more susceptible to change, whereas sites 358 and 762 are less responsive.

Note that the four different glycopeptides have different core-fucosylation ratios using our index formula (Figure 2). This index does not indicate the actual absolute quantity of the core-fucosylation level of those sites. One issue is that trypsin cleavage may be inhibited by the nearby core-fucosylation.[24] Among the four targeted core-fucosylation sites, three sites do not have this problem, whereas site 397 may inhibit trypsin digestion at its N side. Although no missed cleavage was detected, it is difficult to know if there is a missed cleavage. If there is one missed cleavage before site 397, the peptide will contain 43 amino acids, which is beyond the detection limit of our mass spectrometer. Therefore, the core-fucosylation ratio calculated for site 397 does not indicate the actual core-fucosylation level with full digestion. Also, previous studies have shown that different glycopeptides

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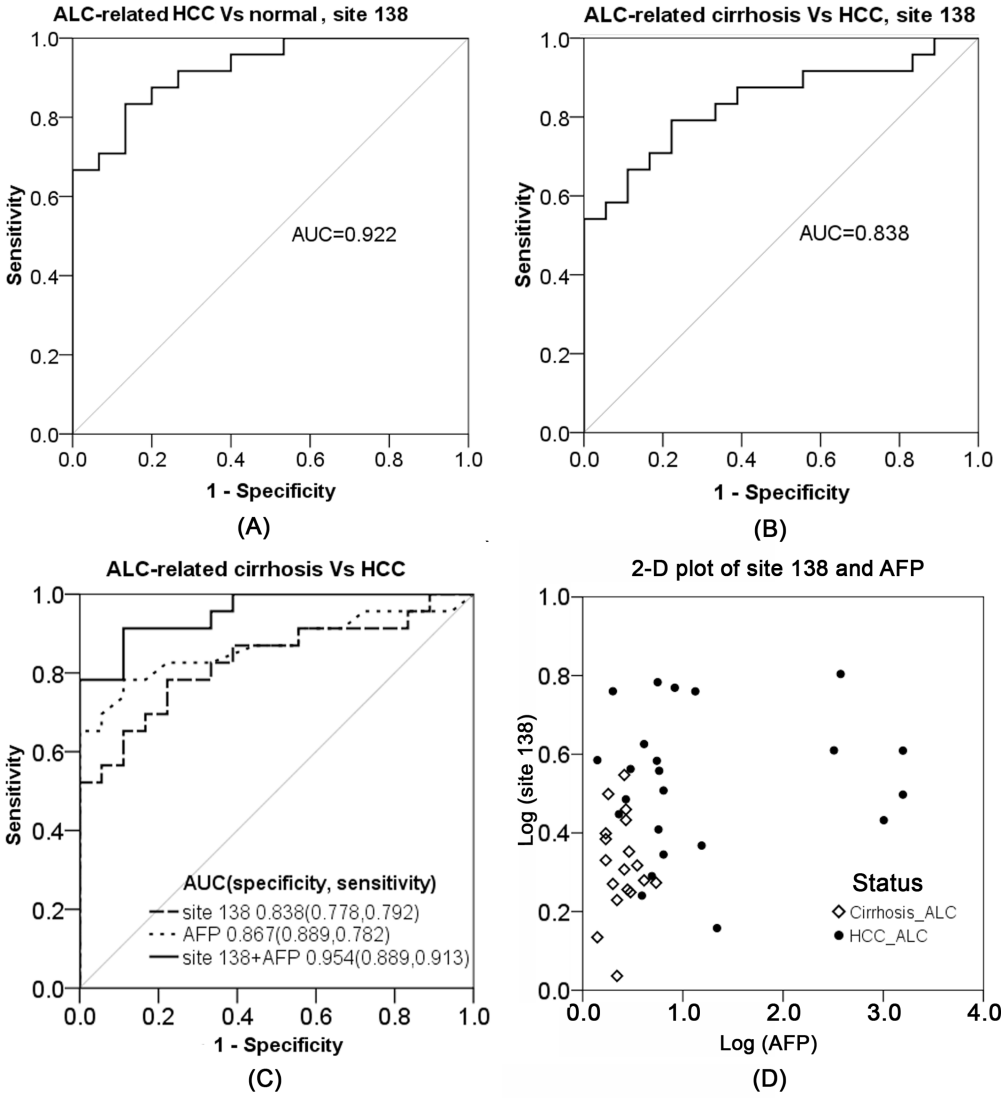

Figure 3. ROC analysis of the core-fucosylation ratio at site 138 between ALC-related HCC and cirrhosis samples (A) and between ALC-related HCC and normal samples (B). (C) Combination analysis of AFP and the core-fucosylation ratio at site 138 between ALC-related cirrhosis and HCC samples. (D) Two-dimensional plot of AFP and core-fucosylation ratio at site 138 between ALC-related cirrhosis and HCC samples.

may have different ionization efficiencies in MS.[23] Equal molar amounts of peptides and glycopeptides with the same peptide backbone have different signal intensities in MS.[23] Therefore, the core-fucosylation ratio calculated in this study does not provide the absolute quantity of the core-fucosylation level of those sites. Nevertheless, all samples in this study were processed following the same procedure so that the level of digestion should be similar among samples, and the response in MS of the same core-fucosylated peptide should be the same among samples. Thus, even when we do not obtain the true ratio, we are still able to use this index to compare different disease states, which is the object of this work.

Core-Fucosylation Ratio of Ceruloplasmin in HCC and Cirrhosis Samples with Different Etiologies

The core-fucosylation changes of ceruloplasmin at different sites were distinctly different in various liver diseases. The core-fucosylation ratio changed dramatically in ALC-related HCC samples but not in HBV- or HCV-related HCC samples (Figures 2, 4 and 5).

Compared to that of normal samples, the core-fucosylation ratio of ceruloplasmin in ALC-related HCC samples was significantly upregulated at all four sites, with $p < 0.001$. When comparing ALC-related cirrhosis with ALC-related HCC samples, the core-fucosylation ratio of three sites was upregulated in HCC samples, with $p < 0.001$ at site 138 and $p < 0.01$ at sites 397 and 762. The level of change was not significantly different for early stage or late stage HCC samples. Early stage and late stage HCC samples were therefore combined for further comparison. The ROC curve analysis between ALC-related HCC samples and normal samples at site 138 resulted in an AUC value of 0.922 with a specificity of 80.0% at a sensitivity of 87.5% (Figure 3A). The ROC curve analysis at site 138 between ALC-related cirrhosis and HCC resulted in an AUC value of 0.838 with a specificity of 77.8% at a sensitivity of 79.2% (Figure 3B). When combining the core-fucosylation ratio of site 138 and the clinical AFP value, as shown in Figure 3C, the AUC value between ALC-related cirrhosis and HCC increased to 0.954 (confirmed by 10-fold cross-validation) ($OR_{site138} = 12.26$, $p = 0.017$; $OR_{AFP} = 3.64$, $p = 0.022$). The model fit was significantly improved over the one with AFP alone (AUC value = 0.867) (LR

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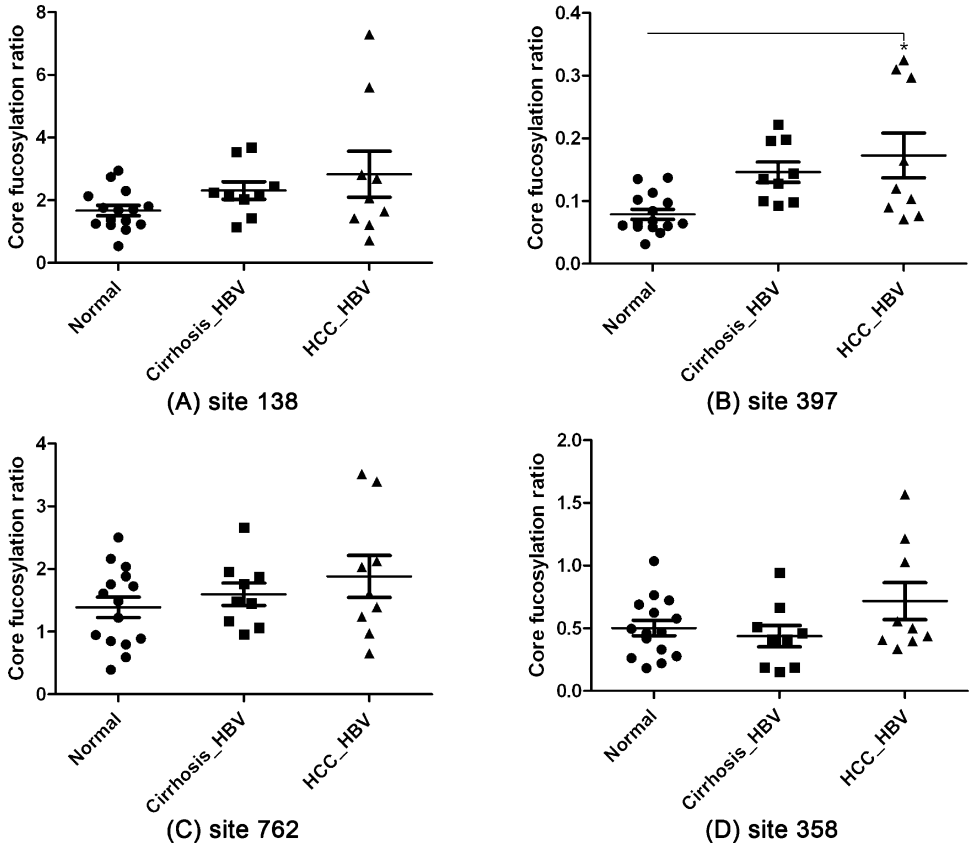

Figure 4. Core-fucosylation ratios of ceruloplasmin at sites 138 (A), 397 (B), 762 (C), and 358 (D) in normal and HBV-related serum samples. A *t*-test comparison was made between each disease group and normal group as well as between cirrhosis and each disease group. Error bar indicates SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

test $p = 0.0002$). This improvement is mainly due to the enhanced sensitivity from 78.2 to 91.3%, as shown in 2D plot (Figure 3D). Here, the optimum sensitivity and specificity of AFP was used instead of that at the 20 ng/mL cutoff that is widely used in clinical practice. The reason is that only 25% of ALC-related HCC samples used in this study have AFP > 20 ng/mL. Therefore, in ALC-related patients, the AFP cutoff should be a lower value than 20 ng/mL. DCP is widely used as a marker in Japan to complement AFP. However, DCP has a poor AUC value in patients with nonviral etiology.[6] The core-fucosylation ratio of ceruloplasmin at site 138 could be a good complementary biomarker of AFP for screening in this population. The ROC curve analysis of the other three sites showed lower AUC values than that at site 138 (Supporting Information Figure S6).

In this study, the charge state with stronger peak intensity that could be detected in all tested samples was chosen for each target peptide. Only one of the four target glycopeptides (site 397) has two charge states in all of the tested samples. The core-fucosylation ratio of charge 2+ peptides with Asn397 has been listed in this article, which has a stronger peak intensity. The analysis of charge 3+ is shown in Supporting Information Figure S7. The core-fucosylation ratio of the two charge states turned out to be somewhat different in our study ($p < 0.05$), where charge 2+ has an ROC value of 0.723 and charge 3+ has an ROC value of 0.813 to distinguish ALC-related cirrhosis and ALC-related HCC, although the correlation of the two is 0.921. Therefore, charge state needs to be considered in biomarker discovery analysis. The ROC value of the core-fucosylation ratio of one peptide to distinguish two disease states is charge-state-specific.

Two other clinical indices, INR and HGB, also showed enhanced AUC values at 0.877 and 0.880, respectively, when combined with the core-fucosylation ratio at site 138 to distinguish ALC-related HCC from ALC-related cirrhosis samples (Supporting Information Figure S8). INR stands for international normalized ratio, a measure of blood coagulation that is based on prothrombin time, and HGB stands for the amount of hemoglobin in blood.

In HBV-related samples, the core-fucosylation ratio of HCC samples at site 397 was higher than that in normal samples, with $p < 0.05$ (Figure 4), whereas HCV-related HCC samples had significant upregulation of core-fucosylation at sites 138 and 397, with $p < 0.05$ and $p < 0.001$, respectively (Figure 5). However, a statistically significant core-fucosylation change was not found between HBV-related cirrhosis and HCC samples or between HCV-related cirrhosis and either early stage HCC or late stage HCC samples.

Pooled Sample Analysis of the Core-Fucosylation Ratio of Ceruloplasmin in Liver Diseases

Samples with the same state regardless of their etiologies were pooled for further analysis (Figure 6), which included 45 cirrhosis samples, 56 HCC samples, and 15 normal samples. The statistical result showed that the core-fucosylation ratios at all four sites were higher in HCC samples than those in normal samples ($p < 0.01$) (Figure 6A). Meanwhile, despite the remarkable difference of the core-fucosylation ratio at site 138 in ALC-related cirrhosis and HCC samples (AUC = 0.838, $p < 0.001$), after mixing samples of all etiologies the core-fucosylation ratio at site 138 appeared to have a much lower

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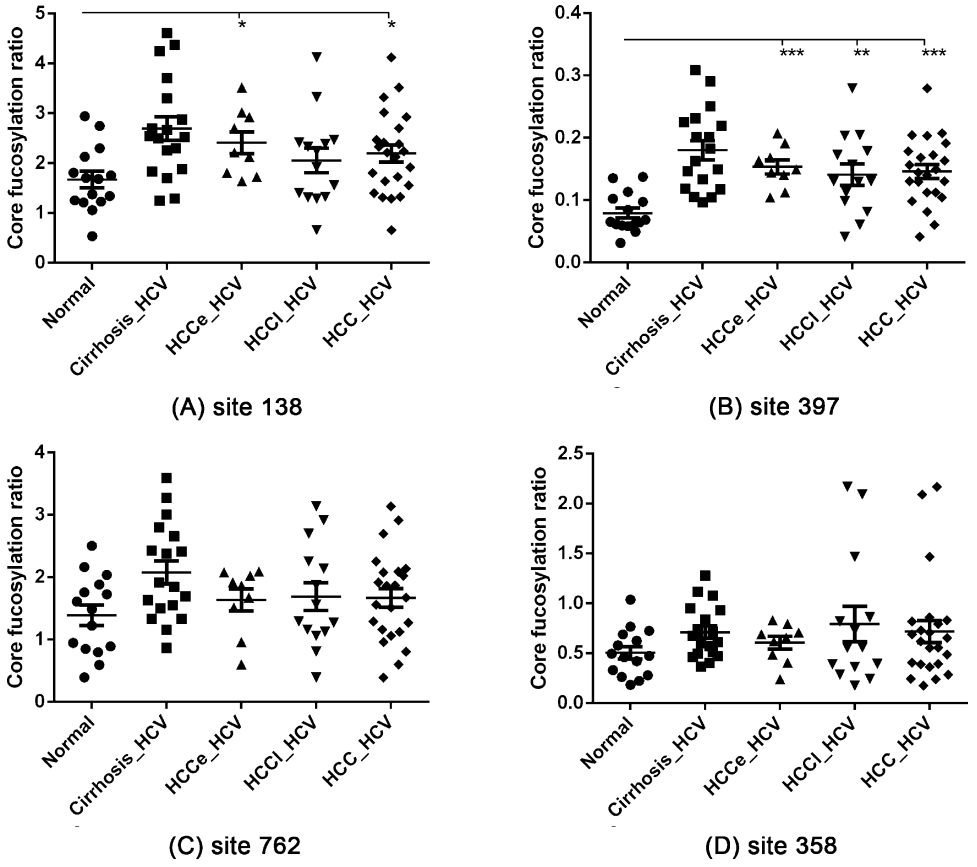

Figure 5. Core-fucosylation ratios of ceruloplasmin at sites 138 (A), 397 (B), 762 (C), and 358 (D) in normal and HCV-related serum samples. HCCe, HCC early stage; HCCl, HCC late stage; HCC, all HCC samples. A *t*-test comparison was made between each disease group and normal group as well as between cirrhosis and each disease group. Error bar indicates SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

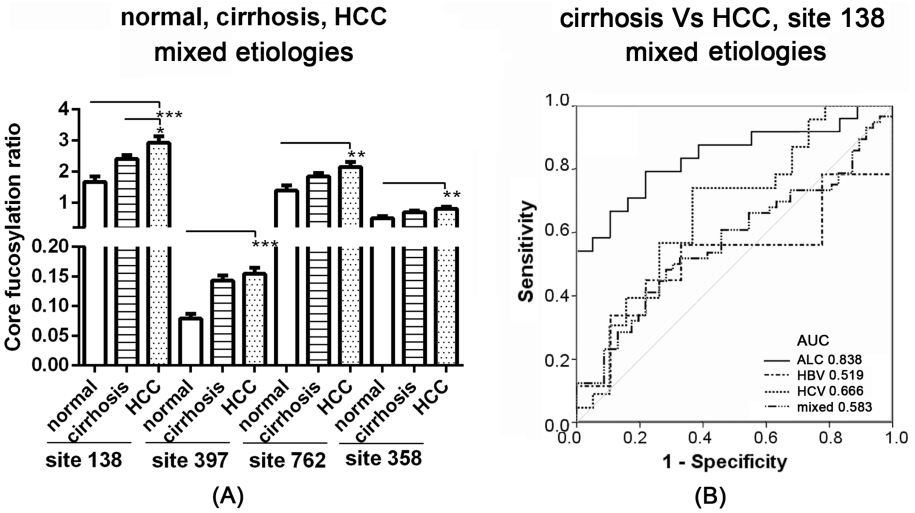

Figure 6. (A) Core-fucosylation ratios of ceruloplasmin at sites 138, 358, 397, and 762 in normal samples and cirrhosis and HCC samples with mixed etiologies. A *t*-test comparison was made between each disease group and normal group as well as between cirrhosis and each disease group. Error bar indicates SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. (B) Receiver operating curve (ROC) analysis of the core-fucosylation ratio at site 138 in cirrhosis and HCC samples with a single etiology and with mixed etiologies.

significance level (AUC = 0.583, $p = 0.034$) between cirrhosis and HCC samples because of the low values in HBV-related samples (AUC = 0.519) and in HCV-related samples (AUC = 0.666) (Figure 6B). No difference was found at other sites. Clinicians usually follow specific etiologies, generally HCV and alcohol related in the U.S. If the etiology is unknown (i.e., whether the patient had HCV or alcohol related), then it might be of limited value given our results on the mixed samples. However, we would expect to see an effect on this site for an unknown that was alcohol related, whereas with HCV we would expect to see a minimal response.

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In terms of marker discovery, it is thus necessary to separate HCC samples with different etiologies to avoid masking potential changes that could serve as markers. The site-specific analysis of the core-fucosylation ratio of ceruloplasmin provides a potentially important marker for early HCC detection in cirrhosis patients with ALC etiology.

Samples with the same etiology (ALC/HBV/HCV) regardless of the disease states were also pooled and analyzed (Supporting Information Figure S9). Alcohol intake and HBV or HCV infection all increased core-fucosylation ratios of ceruloplasmin at most of the sites with different significance levels compared to normal samples.

Other potential markers for HCC were also studied for site-specific core-fucosylation changes, including alpha-2 macroglobulin and transferrin, with similar procedures described above. Neither of these proteins showed a significant difference in the level of core-fucosylation at any of the potential glycosites between cirrhosis and HCC samples with either ALC, HBV, or HCV etiology (data not shown).

CONCLUSIONS

Using a mass-selected site-specific assay, we have found that altered site-specific core-fucosylation of ceruloplasmin can serve as a potential marker for ALC-related HCC. Site 138 of ceruloplasmin, compared with other core-fucosylated sites, is the most highly core-fucosylated and is one of the sites that is most susceptible to change. This site showed the highest AUC value of 0.838 to distinguish ALC-related HCC from ALC-related cirrhosis samples. When combining the core-fucosylation ratio of site 138 and the level of AFP, the AUC value increased to 0.954, which was markedly improved compared to that of AFP alone (AUC = 0.867). However, in HBV- or HCV-related disease samples, although the core-fucosylation ratio at sites 138 and/or 397 was significantly higher than that in normal samples, no difference was found between cirrhosis and HCC samples. When combining samples with the same disease states regardless of their etiologies, the core-fucosylation ratio at site 138 showed a much lower AUC level (0.583) to distinguish between cirrhosis and HCC samples compared with that in ALC-related disease samples (AUC = 0.838). Therefore, the core-fucosylation ratio of ceruloplasmin at site 138 may serve as a potential marker for ALC-related HCC, which is both site-specific and etiology-specific. It would be used in conjunction with other markers for HCC diagnosis. Our findings reveal a new field for future marker discovery: the core-fucosylation change of a specific glycosite rather than of the entire protein may serve as an improved marker for disease diagnosis; meanwhile, markers targeting etiology-specific HCC may improve the specificity and sensitivity.

ASSOCIATED CONTENT

Supporting Information

Workflow of this study; sequences of ceruloplasmin and the identified peptides by MS; SDS-PAGE of ceruloplasmin from immunoprecipitation; MS spectra of core-fucosylated peptides; XIC extraction of targeted peptides listed in Table 2; ROC analysis of core-fucosylation ratio at sites 358, 397, and 762 between ALC-related cirrhosis and HCC samples and between ALC-related HCC and normal samples; ROC analysis of the core-fucosylation ratio of glycopeptides containing site 397 with charge 2+ or charge 3+ in ALC-related cirrhosis and HCC samples; ROC analysis of the core-fucosylation ratio at site 138 combined with INR and HGB in ALC-related cirrhosis and HCC samples; and core-fucosylation ratios of ceruloplasmin at sites 138, 358, 397, and 762 in ALC, HBV, and HCV-related liver disease and in normal serum samples. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

HCC, hepatocellular carcinoma; ALC, alcohol; HBV, hepatitis B virus; HCV, hepatitis C virus; AFP, alpha-fetoprotein; LCA, *Lens culinaris* agglutinin; AAL, *Aleuria aurantia* lectin; AFP-L3, LCA-bound fraction of AFP; A1AT, alpha-1-antitrypsin; IgG, immunoglobulin G; LC-LTQ-MS, liquid chromatography with linear ion trap mass spectrometry; CID, collision ionization dissociation; ROC, receiver operating characteristic; AUC, area under the curve; XIC, extracted ion chromatogram; TCEP, tris (2-carboxyethyl) phosphine; IAA, iodoacetamide; TEAB, triethylammonium bicarbonate; TFA, trifluoroacetic acid; LR test, likelihood ratio test; OR, odds ratio

REFERENCES

(1) Kamangar, F.; Dores, G. M.; Anderson, W. F. Patterns of cancer incidence, mortality, and prevalence across five continents: Defining priorities to reduce cancer disparities in different geographic regions of the world. *J. Clin. Oncol.* **2006**, *24*, 2137−2150.

(2) Llovet, J. M.; Burroughs, A.; Bruix, J. Hepatocellular carcinoma. *Lancet* **2003**, *362*, 1907−1917.

(3) El-Serag, H. B. Hepatocellular carcinoma. *N. Engl. J. Med.* **2011**, *365*, 1118−1127.

(4) Trevisani, F.; D'Intino, P. E.; Morselli-Labate, A. M.; Mazzella, G.; Accogli, E.; Caraceni, P.; Domenicali, M.; De Notariis, S.; Roda, E.; Bernardi, M. Serum alpha-fetoprotein for diagnosis of hepatocellular carcinoma in patients with chronic liver disease: influence of HBsAg and anti-HCV status. *J. Hepatol.* **2001**, *34*, 570−575.

(5) Nakamura, S.; Nouso, K.; Sakaguchi, K.; Ito, Y. M.; Ohashi, Y.; Kobayashi, Y.; Toshikuni, N.; Tanaka, H.; Miyake, Y.; Matsumoto, E.; Shiratori, Y. Sensitivity and specificity of des-gamma-carboxy prothrombin for diagnosis of patients with hepatocellular carcinomas varies according to tumor size. *Am. J. Gastroenterol.* **2006**, *101*, 2038−2043.

(6) Marrero, J. A.; Feng, Z. D.; Wang, Y. H.; Nguyen, M. H.; Befeler, A. S.; Roberts, L. R.; Reddy, K. R.; Harnois, D.; Llovet, J. M.; Normolle, D.; Dalhgren, J.; Chia, D.; Lok, A. S.; Wagner, P. D.; Srivastava, S.; Schwartz, M. Alpha-fetoprotein, des-gamma carboxyprothrombin, and lectin-bound alpha-fetoprotein in early hepatocellular carcinoma. *Gastroenterology* **2009**, *137*, 110−118.

(7) Lok, A. S.; Sterling, R. K.; Everhart, J. E.; Wright, E. C.; Hoefs, J. C.; Di Bisceglie, A. M.; Morgan, T. R.; Kim, H. Y.; Lee, W. M.; Bonkovsky, H. L.; Dienstag, J. L.; Grp, H. C. T. Des-gamma-carboxyprothrombin and alpha-fetoprotein as biomarkers for the early detection of hepatocellular carcinoma. *Gastroenterology* **2010**, *138*, 493−502.

(8) Lin, Z. X.; Yin, H. D.; Lo, A.; Ruffin, M. T.; Anderson, M. A.; Simeone, D. M.; Lubman, D. M. Label-free relative quantification of alpha-2-macroglobulin site-specific core-fucosylation in pancreatic

cancer by LC-MS/MS. *Electrophoresis* [Online early access]. DOI: 10.1002/elps.201300376. Published Online: Dec 27, **2013**.
(9) Ahn, J. M.; Sung, H. J.; Yoon, Y. H.; Kim, B. G.; Yang, W. S.; Lee, C.; Park, H. M.; Kim, B. J.; Kim, B. G.; Lee, S. Y.; An, H. J.; Cho, J. Y. Integrated glycoproteomics demonstrates fucosylated serum paraoxonase 1 alterations in small cell lung cancer. *Mol. Cell Proteomics* **2013**, *13*, 30−48.
(10) Sato, Y.; Nakata, K.; Kato, Y.; Shima, M.; Ishii, N.; Koji, T.; Taketa, K.; Endo, Y.; Nagataki, S. Early recognition of hepatocellular carcinoma based on altered profiles of alpha-fetoprotein. *N. Engl. J. Med.* **1993**, *328*, 1802−1806.
(11) Nakagawa, T.; Miyoshi, E.; Yakushijin, T.; Hiramatsu, N.; Igura, T.; Hayashi, N.; Taniguchi, N.; Kondo, A. Glycomic analysis of alpha-fetoprotein L3 in hepatoma cell lines and hepatocellular carcinoma patients. *J. Proteome Res.* **2008**, *7*, 2222−2233.
(12) Toyoda, H.; Kumada, T.; Tada, T.; Kaneoka, Y.; Maeda, A.; Kanke, F.; Satomura, S. Clinical utility of highly sensitive *Lens culinaris* agglutinin-reactive alpha-fetoprotein in hepatocellular carcinoma patients with alpha-fetoprotein <20 ng/mL. *Cancer Sci.* **2011**, *102*, 1025−1031.
(13) Comunale, M. A.; Rodemich-Betesh, L.; Hafner, J.; Wang, M.; Norton, P.; Di Bisceglie, A. M.; Block, T.; Mehta, A. Linkage specific fucosylation of alpha-1-antitrypsin in liver cirrhosis and cancer patients: implications for a biomarker of hepatocellular carcinoma. *PLoS One* **2010**, *5*, e12419.
(14) Yildirim, A.; Meral, M.; Kaynar, H.; Polat, H.; Ucar, E. Y. Relationship between serum levels of some acute-phase proteins and stage of disease and performance status in patients with lung cancer. *Med. Sci. Monit.* **2007**, *13*, CR195−CR200.
(15) Hanas, J. S.; Hocker, J. R.; Cheung, J. Y.; Larabee, J. L.; Lerner, M. R.; Lightfoot, S. A.; Morgan, D. L.; Denson, K. D.; Prejeant, K. C.; Gusev, Y.; Smith, B. J.; Hanas, R. J.; Postier, R. G.; Brackett, D. J. Biomarker identification in human pancreatic cancer sera. *Pancreas* **2008**, *36*, 61−69.
(16) Walker, C.; Gray, B. N. Acute-phase reactant proteins and carcinoembryonic antigen in cancer of the colon and rectum. *Cancer* **1983**, *52*, 150−154.
(17) Liu, Y. S.; He, J. T.; Li, C.; Benitez, R.; Fu, S.; Marrero, J.; Lubman, D. M. Identification and confirmation of biomarkers using an integrated platform for quantitative analysis of glycoproteins and their glycosylations. *J. Proteome Res.* **2010**, *9*, 798−805.
(18) El-Serag, H. B. Epidemiology of viral hepatitis and hepatocellular carcinoma. *Gastroenterology* **2012**, *142*, 1264−1273.
(19) Morgan, T. R.; Mandayam, S.; Jamal, M. M. Alcohol and hepatocellular carcinoma. *Gastroenterology* **2004**, *127*, S87−96.
(20) Hussain, S. P.; Schwank, J.; Staib, F.; Wang, X. W.; Harris, C. C. TP53 mutations and hepatocellular carcinoma: insights into the etiology and pathogenesis of liver cancer. *Oncogene* **2007**, *26*, 2166−2176.
(21) Tarentino, A. L.; Plummer, T. H. Enzymatic deglycosylation of asparagine-linked glycans: purification, properties, and specificity of oligosaccharide-cleaving enzymes from *Flavobacterium meningosepticum*. *Methods Enzymol.* **1994**, *230*, 44−57.
(22) Huttenhain, R.; Surinova, S.; Ossola, R.; Sun, Z.; Campbell, D.; Cerciello, F.; Schiess, R.; Bausch-Fluck, D.; Rosenberger, G.; Chen, J. C.; Rinner, O.; Kusebauch, U.; Hajduch, M.; Moritz, R. L.; Wollscheid, B.; Aebersold, R. N-Glycoprotein SRMAtlas: a resouce of mass spectrometeric assays for N-glycosites enabling consistent and multiplexed protein quantification for clinical applications. *Mol. Cell. Proteomics* **2013**, *12*, 1005−1016.
(23) Stavenhagen, K.; Hinneburg, H.; Thaysen-Andersen, M.; Hartmann, L.; Silva, D. V.; Fuchser, J.; Kaspar, S.; Rapp, E.; Seeberger, P. H.; Kolarich, D. Quantitative mapping of glycoprotein micro-heterogeneity and macro-heterogeneity: an evaluation of mass spectrometry signal strengths using synthetic peptides and glycopeptides. *J. Mass Spectrom.* **2013**, *48*, 627−639.
(24) Deshpande, N.; Jensen, P. H.; Packer, N. H.; Kolarich, D. GlycoSpectrumScan: fishing glycopeptides from MS spectra of protease digests of human colostrum sIgA. *J. Proteome Res.* **2010**, *9*, 1063−1075.